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Principles of Safety Pharmacology

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Preface

Safety pharmacology has evolved from a mixture of toxicological investigations to what we now recognize as a frontloaded integrated risk assessment during the 20 years that has followed the recognition of rare but potentially lethal adverse drug reactions, exemplified by terfenadine-induced torsades de pointes. Safety pharmacology is most important during the period of preclinical drug discovery and development. Safety pharmacology has evolved into an astute and flexible discipline and now paradoxically leads the way in discovery standardization by virtue of the efforts that have taken place to validate preclinical methods. Numerous examples exist where a collection of positive and negative controls are used to template a method—an approach rarely reciprocated in such detail and with such diligence in Discovery pharmacology.

In this volume, we have assembled reviews of all the main aspects of preclinical and translational safety pharmacology, with emphasis on explanation for choice of approach and the testing of validity. The articles are intended to serve as reference for industry and text for the growing undergraduate and postgraduate programs and courses on safety pharmacology that are emerging in universities worldwide.

Raritan, NJ, USA — Michael K. Pugsley
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Contents

Part I

An Overview of Safety Pharmacology and Its Role in Drug Discovery

A Historical View and Vision into the Future of the Field of Safety Pharmacology

Alan S. Bass, Toshiyasu Hombo, Chieko Kasai, Lewis B. Kinter, and Jean-Pierre Valentin

"1. Don't do something just because you can.
2. Don't do something just because it has always been done.
3. Don't do something just because others do it."
"4. Don't do something because (you believe) it is expected.
5. Don't do something the results of which cannot be interpreted.
6. Do something because there is a reasonable expectation it will provide knowledge necessary for an accurate decision."

Gerhard Zbinden and Robert Hamlin (Hamlin 2006)

Contents

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Abstract

Professor Gerhard Zbinden recognized in the 1970s that the standards of the day for testing new candidate drugs in preclinical toxicity studies failed to identify acute pharmacodynamic adverse events that had the potential to harm participants in clinical trials. From his vision emerged the field of safety pharmacology, formally defined in the International Conference on Harmonization (ICH) S7A guidelines as "those studies that investigate the potential undesirable pharmacodynamic effects of a substance on physiological functions in relation to exposure in the therapeutic range and above." Initially, evaluations of small-molecule pharmacodynamic safety utilized efficacy models and were an ancillary responsibility of discovery scientists. However, over time, the relationship of these studies to overall safety was reflected by the regulatory agencies who, in directing the practice of safety pharmacology through guidance documents, prompted transition of responsibility to drug safety departments (e.g., toxicology). Events that have further shaped the field over the past 15 years include the ICH S7B guidance, evolution of molecular technologies leading to identification of new therapeutic targets with uncertain toxicities, introduction of data collection using more sophisticated and refined technologies, and utilization of transgenic animal models probing critical scientific questions regarding novel targets of toxicity. The collapse of the worldwide economy in the latter half of the first decade of the twenty-first century, continuing high rates of compound attrition during clinical development and post-approval and sharply increasing costs of drug development have led to significant strategy changes, contraction of the size of pharmaceutical organizations, and refocusing of therapeutic areas of investigation. With these changes has come movement away from dedicated internal safety pharmacology capability to utilization of capabilities within external contract research organizations. This movement has created the

opportunity for the safety pharmacology discipline to come "full circle" and return to the drug discovery arena (target identification through clinical candidate selection) to contribute to the mitigation of the high rate of candidate drug failure through better compound selection decision making. Finally, the changing focus of science and losses in didactic training of scientists in whole animal physiology and pharmacology have revealed a serious gap in the future availability of qualified individuals to apply the principles of safety pharmacology in support of drug discovery and development. This is a significant deficiency that at present is only partially met with academic and professional society programs advancing a minimal level of training. In summary, with the exception that the future availability of suitably trained scientists is a critical need for the field that remains to be effectively addressed, the prospects for the future of safety pharmacology are hopeful and promising, and challenging for those individuals who want to assume this responsibility. What began in the early part of the new millennium as a relatively simple model of testing to assure the safety of Phase I clinical subjects and patients from acute deleterious effects on life-supporting organ systems has grown with experience and time to a science that mobilizes the principles of cellular and molecular biology and attempts to predict acute adverse events and those associated with long-term treatment. These challenges call for scientists with a broad range of in-depth scientific knowledge and an ability to adapt to a dynamic and forever changing industry. Identifying individuals who will serve today and training those who will serve in the future will fall to all of us who are committed to this important field of science.

Keywords

Safety pharmacology • Cardiovascular system • Central nervous system • Peripheral nervous system • Respiratory system • INTERNATIONAL CONFERENCE ON HARMONIZATION • ICH S7A • ICH S7B • ICH E14 • United States Food and Drug Administration • European Medicines Agency • Japan Pharmaceutical and Medicines Devices Agency

List of Abbreviations

ABPI	Association of the British Pharmaceutical Industry
ADRs	Adverse Drug Reactions
AEs	Adverse Events
APD	Action Potential Duration
BfArM	Bundesinstitut für Arzneimittel und Medizinprodukte which is the Federal Institute for Drugs and Medical Devices
CFR	Code of Federal Regulations
CiPA	Comprehensive In vitro Proarrhythmia Assay
CNS	Central Nervous System
CPMP	Committee for Proprietary Medicinal Products

CROs	Contract Research Organizations
CSRC	Cardiac Safety Research Consortium
DSP	Diplomate in Safety Pharmacology
ECG	Electrocardiogram
ECVAM	European Centre for the Validation of Alternative Methods
EFPIA	European Federation of the Pharmaceutical Industry Association
eIND	Exploratory Investigational New Drug Application
EMEA	European Medicines Agency
EU	European Union
EWG	Expert Working Group
FDA	United States Food and Drug Administration
GLP	Good Laboratory Practice
hERG	human Ether-a-go-go-Related Gene
ICH	International Conference on Harmonization
ILSI	International Life Sciences Institute
IWG	Implementation Working Group
HESI	Health and Environmental Sciences Institute
IND	Investigational New Drug Application
iPSCs	Induced pluripotent stem cells
JACL	Japan Association of Contract Laboratories for Safety Evaluation
JNDA	Japanese New Drug Applications
JPMA	Japanese Pharmaceutical Manufacturers Association
MHLW	Ministry of Health, Labour and Welfare
MHW	Ministry of Health and Welfare
NCEs	New Chemical Entities
NDAs	New Drug Applications
PhRMA	Pharmaceutical Research and Manufacturers of America
Q&As	Questions and Answers
QT	Duration of the QT interval of the cardiac electrocardiogram
QT PRODACT	QT Interval Prolongation: Project for Database Construction
R&D	Research and Development
SEND	Standard for Exchange of Nonclinical Data
SP	Safety pharmacology
SPS	Safety Pharmacology Society
JSPS	Japanese Safety Pharmacology Society
TDP	Therapeutic Products Directorate
TQT	Clinical Thorough QT study
USA	United States of America

Professor Gerhard Zbinden argued that the major clinical endpoints related to safety in early human trials were not adequately evaluated in the routine animal safety studies being carried out in the 1970s, where the focus was on pathomorphological

and lab parameters appearing late during treatment, while damages of bodily functions appear early. This different focus posed a significant and underappreciated risk to healthy normal volunteers and patients participating in early clinical evaluations of new drugs (Zbinden 1979). Zbinden's hypothetical "gap" was dramatically exposed in the mid-1990s, when it became apparent that individuals were being placed at an unacceptable risk of cardiac toxicity and death from drugs that were marketed for treatment of a variety of non-life-threatening diseases (Shah 2002b). In response, the fledgling field of safety pharmacology was formalized in international regulatory guidance, marking rapid recognition of its contributions to protecting clinical trial subjects (Bass et al. 2004b, 2011). In the intervening years, advances in science and technology and contributions from regulators, scientists, and the public have challenged safety assessment of new drugs, and safety pharmacology in particular, to evolve quickly, sometimes ahead of scientific consensus and governing regulations. Added to this landscape are the growing economic challenges and a business model for the discovery and development of new drugs that many claim is not sustainable as evidenced by the higher difficulties of bringing new drugs to market, despite continuous attempts to alter the model to increase the probability of success (Hay et al. 2014; Holdren et al. 2012; Urban et al. 2014).

Accounting for the relatively brief history of safety pharmacology, the authors have laid out a review of the discipline, from the time of Dr. Zbinden to the present day, as well as forecasting the future from their vantage points of leaders deeply committed and involved in the growth of the field. The periods covered in this chapter include the time prior to adoption by the International Conference on Harmonization (ICH) the topics of guidelines which would ultimately govern the regulatory practice of safety pharmacology, the trials, tribulations, and constantly evolving challenges associated with the implementation of the laboratories conforming with those guidelines and the scientific and intellectual growth and maturation of the field that was aligning and adapting to the changing scientific and regulatory landscape and business environment of the pharmaceutical industry. The chapter concludes with thoughts on the future challenges faced by safety pharmacology and the scientists that will shepherd the continued evolution of this discipline, as those scientists will also be expected to anticipate and respond to the events that will unfold over the coming years.

1 Prior to Adoption of ICH S7: Safety Pharmacology/General Pharmacology

Like any other profession or scientific discipline, safety pharmacology has its beginnings, in terms of name, concepts, discipline, practices, philosophy, and specific tests. Gerhard Zbinden (1979) is generally credited with calling attention to the "disconnect" between the study endpoint (e.g., histopathology) of standard nonclinical toxicological test procedures of that era and the types of adverse drug reactions (ADRs) observed by clinicians in clinical trials: that whereas the former

focused heavily upon morphological and biochemical lesions, the latter were focused on organ functional side effects. Further, in an era when clinical chemistry and histopathology were dominant in nonclinical safety testing, Zbinden raised the specter that potentially life-threatening functional side effects of concern to physicians and patients could be discovered only late in standard toxicological testing. Zbinden's warning was dramatically substantiated in the mid-1990s with the recognition of drug-related "long-QT" syndrome and risk of a potentially fatal ventricular tachyarrhythmia (Anon 2005a, 2014; Bass et al. 2005, 2007, 2008; Borchert et al. 2006; Darpo 2010; Darpo et al. 2006; Kinter et al. 2004; Shah 2002a, b, 2007). Thus, there can be little debate that G. Zbinden is the "father" of what is known today as modern safety pharmacology. Ironically, Zbinden was also an advocate of the value of rat models for cardiovascular assessments of drugs, but we now recognize that this rodent species is an inappropriate model with which to detect drug-induced long-QT effects because the rat relies on a different cardiac delayed-rectifying potassium current (I_{Kr}) for cardiac repolarization than that used by humans (see below).

The first explicit references to safety pharmacology in regulatory guidances for investigations of potential for undesirable pharmacological activities in pharmaceutical research and development (R&D) appeared in ICH documents and subsequent FDA release of the ICH S6 guidance document in July 1997: '*Safety Pharmacology studies measure functional indices of potential toxicity. … The aim of the Safety Pharmacology studies should be to reveal any functional effects on the major physiological systems* (e.g., *cardiovascular, respiratory, renal, and central nervous systems).*' (Anon 2012a, b), and '*Safety Pharmacology includes the assessment of effects on vital functions, such as cardiovascular, central nervous, and respiratory systems, and these should be evaluated prior to human exposure*' (Anon 1997b, c). These "original concepts" of safety pharmacology were subsequently codified in separate ICH guidance documents ICH S7A (Anon 2001c, e) and ICH S7B (Anon 2005a, b) and established safety pharmacology as it applies to the development of new pharmaceutical agents today (Fig. 1).

What is uncertain is the origin of the term "safety pharmacology" within the context of the ICH guidance. In prior regional guidance documents, the concepts framed and subsequently fleshed out in the 1997 and 2000 ICH documents included components embedded in "general pharmacology" studies (Lumley 1994) and in a description of "pharmacological toxicity" testing (Williams 1990). While Kinter et al. (1994) listed the term "safety pharmacology" as one of several then currently in use to identify investigations of "effects of a new drug on pharmacological targets and organ functions, other than those for which the drug was intended," one of those authors (LK) recalls it was included because safety pharmacology was being used in then early drafts of the 1996 ICH documents. Dr. Gerd Bode, a member of the ICH S7A Expert Working Group (EWG, Table 1), recalls that in the early 1990s ICH defined three disciplines for which guidelines should be drafted: quality, safety, and efficacy. Safety in the original ICH sense was preclinical safety, or preclinical toxicology (i.e., nonclinical testing for unexpected adverse events). Dr. Bode recalls that at that time investigations for adverse functional effects as part

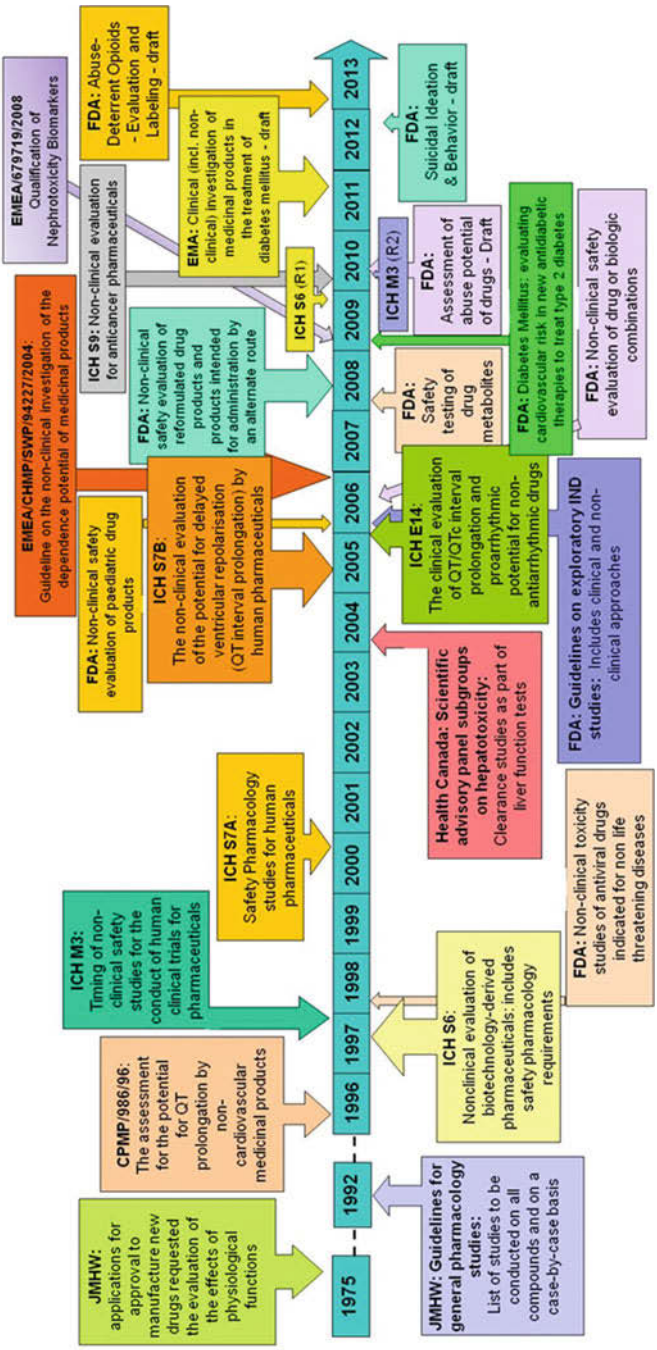

Fig. 1 Scope and implementation date of regulatory guidance documents referring entirely or in part to safety pharmacology over the last 40 years. Over the last decade, there has been an increase in the number and scope of regulatory guidance referring to safety pharmacology endpoints reflecting increasing regulatory concerns. *FDA* United States Food and Drug Administration, *ICH* International Conference on Harmonization, *JMHW* Japanese Ministry of Health and Welfare, *EMEA* European Agency for the Evaluation of Medicinal Products, *CPMP* Committee on Proprietary and Medicinal Products, *CHMP* Committee on Medicinal Products for Human Use

Table 1 ICH-S7A Expert Working Group members

Party	Experts	
MHW	Kannosuke Fujimori (OPSR)[a]	Yoichi Sato (MDEC)
JPMA	Munehiro Hashimoto (Pharmacia and Upjohn)[b] Hiroshi Mayahara (Takeda)	Toshiyasu Hombo (Fujisawa)
EU	Klaus Olejniczak (BfArM)	
EFPIA	Gerd Bode (HMR)	Andrew Sullivan (GW)
FDA	Joseph DeGeorge (CDER)	Martin Green (CBER)
PhRMA	James Moe (Pharmacia and Upjohn) Kenneth Ayers (GW)	Richard Robertson (DuPont)
EFTA	Jurg Seiler (IKS)	
Canada	Peter Grosser (Health Canada)	

[a]Rapporteur from Step 2 through Step 4
[b]Rapporteur from Step 0 though Step 2 sign-off
JMHW Japanese Ministry of Health and Welfare, *JPMA* Japanese Pharmaceutical Manufacturers Association, *EU* European Union, *EFPIA* European Federation of Pharmaceutical Industry Association, *FDA* United States Food and Drug Administration, *PhRMA* Pharmaceutical Research Manufacturers Association, *EFTA* European Free Trade Association, *OPSR* Organization for Pharmaceutical Safety and Research, *MDEC* Medical Device Evaluation Committee, *P&U* Pharmacia and Upjohn, *BfArM* German Federal Institute for Drugs and Medical Devices, *HMR* Hoechst Marion Roussel, *GW* Glaxo Wellcome, *CDER* Center for Drug Evaluation and Research, *CBER* Center for Biologic Evaluation and Research, *IKS* Swiss Kontrollstelle fur Heilmittel

of then "general pharmacology" investigations were redefined incorporating the ICH safety definition; hence "safety pharmacology" appeared first in draft versions of the ICH S6 guideline in 1995. Thus, the term "safety pharmacology" appears to arise de novo in the early 1990s as an amalgamation of the then current general pharmacology terminology and new ICH definition for safety guidance in pharmaceutical development.

Also unclear is why the new term "safety pharmacology" was deemed necessary when "general pharmacology" was both inclusive and common in both regulatory and industry parlance. The regional regulatory guidance that predated the 1997 ICH guidance defined general pharmacological studies as those that revealed both potential useful and harmful properties of a drug in a quantitative manner which permits an assessment of therapeutic risk (Australian NDF4 guidelines, see Lumley, 1994). Williams (1990) referred to general pharmacological properties and pharmacological profiling of candidate drugs that result in unintended or undesirable effects as "pharmacological toxicity." The general guidance included in the Japanese Guidelnes for Toxicity Studies for Drugs (Anon 2001b; an English version of the guidance published by Anon 1995) recommended specific general pharmacology studies to be conducted on all investigational drugs (List A) and additional studies to be conducted "when necessary" (List B). In a paper entitled "The Role of Pharmacological Profiling in Safety Assessment," reviewing the Japanese Lists A and B, Kinter et al. (1994), the authors identified two separate categories of tests: "A. . .test in which the drug is administered to an intact or acutely-prepared animal model for the purpose of assessing the adverse events

. . .(safety profiling)" and a ". . . test in which a drug is evaluated for (1) affinity for a pharmacological target, (2) activity to stimulate, inhibit, . . .(3) activity to stimulate, potentiate,. . . activity of another drug, or (4) activity to stimulate, potentiate, . . . physiological or pharmacological responses. . . (pharmacological profiling)." They further observed that safety profiling (which they labeled "safety pharmacology") was limited to those organ systems of critical interest to primary care physicians (cardiovascular, respiratory, central nervous system (CNS), renal and gastrointestinal) and contributed directly to drug discovery, risk assessment, and patient management, whereas pharmacological profiling (labeled "general pharmacology") cataloged mechanisms by which drugs might impact an organism and were limited only by imagination and available resource. These concepts were further refined in ICH S7A (Anon 2001c, e) to specify drug effects upon the intended pharmacological target (primary pharmacology), drug effects on targets other than the primary target (secondary pharmacology), and drugs effects that adversely impact critical organ functions (safety pharmacology), the definitions in general use today. Thus, the "new" term, safety pharmacology, was needed to delineate the concepts of pharmacologically based toxicity (or safety profiling) from pharmacological profiling, congruent with Dr. Bode's recollection of the term itself (see above).

Functions conducting general pharmacology and/or safety pharmacology studies were distributed across research (discovery) and development (e.g., toxicology) organizations in different companies and viewed the primary value of those investigations as supporting additional/alternative therapeutic applications and/or detection of potential safety hazards (see Williams 1990). This dichotomy of purpose was reflected in the name of an informal pharmaceutical industry trade group of that era—the General Pharmacology/Safety Pharmacology Discussion Group [the progenitor of the current Safety Pharmacology Society (Bass et al. 2004b)]. However, by the time of adoption of the ICH S7A and ICH S7B guidelines (described later in this chapter), the functional responsibilities for safety pharmacology became better defined. In surveys of industry practices carried out by the newly incorporated Safety Pharmacology Society in 2005 and again in 2008, the majority of work across the industry was found in toxicology departments responsible for regulatory studies complying with Good Laboratory Practice (GLP) (Friedrichs et al. 2005; Lindgren et al. 2008; Valentin et al. 2005).

Kinter and Dixon (1995) described a safety pharmacology program for pharmaceuticals wherein they advocated for a tiered approach to testing drug effects on major organ functions:

- Core: cardiovascular, neurological and neuromuscular, respiratory, and renal that are of greatest interest to clinicians
- Special: ocular and auditory functions that address specific pharmacological or chemical class issues
- Ancillary: gastrointestinal, autonomic, and behavioral and drug interactions that satisfy then divergent regional regulatory requirements

Williams (1990) posited that acute or single-dose studies were generally sufficient and that doses selected for pharmacological profiling should "span the

pharmacological and toxicological range in order to provide data on effects occurring at therapeutic as well as potentially toxic levels of exposure." The Kinter and Dixon (1995) paper expanded those concepts to include conduct of safety pharmacology studies to support Phase I clinical trials in humans. This was a fundamental shift from the then current Japanese guidelines that required such studies only prior to registration (Anon 1995). The use of unanesthetized animals and clinical route of administration in order to model the dose route in the single ascending dose phase in healthy normal volunteers, assessment of test article exposure in safety pharmacology studies, and conduct of core safety pharmacology studies in compliance with GLP (Anon 2004b, 2000b) regulations were also advocated by Kinter and Dixon (1995), although the latter was first presented in a European regulatory guidance note (Anon 2004b). Also presented was a new objective: "to identify organ function markers of efficacy and toxicity for support of early clinical studies in humans" (e.g., safety pharmacology biomarkers). In a subsequent paper, the use of cardiovascular telemetry for safety pharmacology evaluations in conscious animals was first described (Kinter et al. 1997). It is noteworthy that the journal *Drug Development Research*, Volume 32 (1994), contains several papers delineating then current practices in cardiovascular, CNS, respiratory, and renal safety pharmacology and results of the first comprehensive industry safety pharmacology survey. All of these concepts were subsequently included at least in part in ICH S7A (Anon 2001c, e).

A final "origin" is that of the specific testing paradigms included in the Japanese general pharmacology guidelines Lists A and B (Anon 1995) and by Williams (1990) as these predate the concepts of pharmacological toxicity, safety profiling, and safety pharmacology (see above). Williams (1990) states that "Typically a battery of 30–40 specialized pharmacological tests is conducted to support drug registration in Japan. Such testing is performed on all classes of pharmaceutical agents, regardless of therapeutic class." One of the current authors (LK) concurs with this statement based upon his review of regulatory study packages presented for registration in Japan during the late 1980s. Those "specialized pharmacological tests" were the in vivo and in vitro bioassays used by pharmacologists to identify potentially useful pharmacological activities before they were replaced by in vitro studies of efficacy (on-target) and off-target sites employing molecular interaction (e.g., ligand–receptor binding assays) screens in the late 1970s. The transition of laboratory practices to the principles of safety pharmacology was intended to focus work of safety scientists on a core of organ functions that were viewed as important to human safety and away from the broad general requirements of the Japanese general pharmacology guidelines, which at the time was of concern to the pharmaceutical industry.

Implementation of safety pharmacology programs compliant with current guidances came about as the transition of carrying out "ad hoc" general pharmacology bioassays of small molecules and biologics following tailored protocols as an ancillary activity of discovery laboratories, to a concerted responsibility of safety pharmacology programs to identify those pharmacodynamic properties with the potential to place clinical trial subjects and patients at risk (Bass et al. 2004a). This focused pharmacodynamic testing began in the early to late 1990s with the appearance of a minimal number of safety pharmacology programs in the United States of

America (USA) and Europe Union (EU) and expanded to, in the first several years following adoption of ICH S7A (2001), a greater number of institutions with established Departments of Safety Pharmacology (Lindgren et al. 2008). Programs in safety pharmacology in Japan were well established and preceded the adoption of the ICH guidelines as a result of the Japanese requirements for general pharmacology. The transition from an "ad hoc approach" to a systematic series of pharmacodynamic assays of the major organ system functions, originally framed in the draft guidances of EU, Japan, and USA (Bass et al. 2004a), led to a Step 0 ICH document on safety pharmacology, which ushered in the beginning of deliberations to define the guidances, ICH S7A and ICH S7B.

2 Eight Years of Deliberations Leading to Step 4 of Two Guidances: Insights into the Expert Working Groups (EWG) Responsible for ICH S7A and ICH S7B Guidances

The mission of the ICH is ". . . to make recommendations towards achieving greater harmonisation in the interpretation and application of technical guidelines and requirements for pharmaceutical product registration, thereby reducing or obviating duplication of testing carried out during the research and development of new human medicines. . .." ICH was established in 1990 and the reader is directed to its website (http://www.ich.org) and the recent publication (van der Laan and DeGeorge 2013) to learn more about the workflow followed by the respective EWGs, who were given the responsibility of crafting two separate guidance documents governing the practice of safety pharmacology.

The development of the international regulatory guidelines concerning safety pharmacology encompassed the period from the evolution of the Step 0 document in 1997 to the final Step 4 document, ICH S7A in 2000, and the emergence of a new topic specific to detecting proarrhythmic risk associated with QT prolongation, with a Step 0 document, ICH S7B in 2000 to the final Step 4 document in 2005. Regional adoption of each of the guidances occurred in the same or following year in the USA and EU, but the adoption of the guidelines in Japan took longer, especially in the case of ICH S7B. In Japan, the ICH S7A guidance went into effect in 2001, but was not fully implemented until 2003 to allow institutions time to establish the necessary GLP compliant capabilities (Valentin et al. 2005). Although the laboratories in Japan had extensive experience with the technical aspects of carrying out the core studies required by the ICH S7A Safety Pharmacology guideline as a result of having worked under the requirements for Japanese General Pharmacology guidance (Anon 1995), the requirement for conformance with GLPs required additional time. With the adoption of ICH S7A in Japan, the Japanese general pharmacology guideline was formally retired. The implementation of the ICH S7B guidance was delayed until 2009 to accommodate the timeframe needed for the implementation of the clinical guidance on assessing QT interval prolongation, ICH E14 in Japan. The events and timing leading up to the respective Step 4 documents are chronicled below.

2.1 S7A Safety Pharmacology Studies for Human Pharmaceuticals (1998–2000)

The topic to develop harmonized guidelines on the practice of safety pharmacology was proposed to the ICH—Steering Committee by the Japanese delegates (Japanese Pharmaceutical Manufacturers Association (JPMA) and Ministry of Health and Welfare [MHW; now referred to as the Ministry of Health, Labour and Welfare (MHLW)], in 1997, and adopted as the Topic S7 in 1998. The membership of the ICH S7 EWG and a chronicle of the timelines and milestones are presented in Tables 1 and 2, respectively.

The first meeting was held in Brussels in March 1999, where the EWG assembled to consider the Step 0 document. The Step 0 document was a compilation of the major principles held in the draft working documents of the participating nations (Bass et al. 2004a). Thereafter, the draft document advanced to a sign-off of the Step 2 version in the fourth EWG meeting in Tokyo in March 2000. In accordance with the ICH process, achieving Step 2 signaled the transition of the role of rapporteur from the pharmaceutical industry member to the regulatory member of the EWG. Since the original recommendation for the ICH topic was made by the JPMA and MHW, the responsibility of rapporteur fell to Dr. Kannosuke Fujimori, the MHW member. Also in accordance with the process laid out by the ICH, an additional milestone of achieving Step 2 was that this was the only time that the pharmaceutical industry members of the EWG have signatory responsibility for the draft ICH document. On the other hand, responsibility for content, scientific background, and strategies continued throughout the whole drafting process for both parties (regulators and industry), and this common responsibility was (independent of signatures) assured via the ICH Steering Committee. At Step 4, only the regulatory members of the ICH EWG serve as signatories to the final ICH document. Step 4 of ICH S7 was achieved in the sixth EWG meeting in San Diego in November 2000. For a more detailed description of the recommendations of ICH 7 (which became ICH S7A at the time of Step 4 adoption; this was to accommodate diverging interpretations within the EWG

Table 2 Chronology of ICH S7A Expert Working Group (EWG) meetings

EWG meeting	Date	Place	Step
First	March 1999	Brussels	1
Second (extra)	August 1999	Tokyo	1
Third	October 1999	Washington, DC	1
Fourth	March 2000	Tokyo	2
Fifth (extra)	September 2000	Bern	3
Sixth (ICH-5)	November 2000	San Diego	4

Note: Extra refers to two meetings held by the ICH S7A EWG that were outside of the regularly scheduled meetings of the ICH Steering Committee; ICH-5 was the fifth conference of ICH that had taken place since ICH was established in 1990; the reader is referred to the ICH website for a definition of the ICH Process (http://www.ich.org)

to recommend guidelines on the study of cardiac ventricular repolarization, which as a result became a new topic designated ICH S7B), the reader is referred to the chapter "Safety Pharmacology: A Practical Guide" (Bass and Williams 2003).

That the ICH S7A document could reach Step 4 in the short time period of only 1 year and 8 months was unprecedented and attributed, in part, to the quality of the Step 0 document that reflected the collective positions of each of the tripartite regulatory members: Guideline for Safety Pharmacology Study by the Japanese MHW, Concept paper on nonclinical safety pharmacology studies by the USA Food and Drug Administration (FDA), and Note for Safety Pharmacology Studies in Medical Products Development by the European CPMP, see Bass et al. (2004a).

2.2 Hierarchy of Organ Systems, Categorization of Safety Pharmacology Studies, and GLP Compliance

As described earlier, the "General Pharmacology Study Guideline" established by MHW in 1991 was the only guideline recognized across the pharmaceutical industry that came close to the present day guidance for safety pharmacology (Anon 1991, 1995). This guideline did not require formal and full compliance with GLP, but did require data collection conforming with the Japanese system of "raw data check," which was a level of documentation that allowed reconstruction of a study by the regulator. The Japanese guidelines clearly specified more than 10 types of bioassays encompassing the evaluation of seven different systems, including general activity and behavior, CNS, autonomic nervous system and smooth muscle, respiratory and cardiovascular systems, digestive system, water and electrolyte metabolism, and other organ systems in which activity would be expected based on class- or chemotype-related pharmacodynamic effects from studies of related drugs (Anon 1991, 1995). These studies were referred to as category A studies and were expected for advancing all new test agents into early clinical trials in Japan (Anon 1995), although the study data itself were not reviewed by the Japanese regulators until the time of the JNDA.

In the first meeting in Brussels in 1999, it was unanimously agreed that safety pharmacology studies should be conducted in compliance with GLP, as was the standard for other nonclinical ICH safety guidances (Anon 2004b, 2000b). Most of the discussions in the subsequent EWG meetings were spent deliberating over the necessity of studying specific organ systems, study objectives, and the designs and parameters used in the evaluation of new molecular entities, primarily small molecules.

The concept of "Hierarchy of Organ Systems" was introduced where three organ systems, i.e., the cardiovascular, respiratory, and central nervous systems of which functions are acutely critical for life, were considered to be the most important to assess as the safety pharmacology battery. The study of each of these organ systems was to be conducted with all test agents, irrespective of their targeted indication or chemical class and they were referred to as the "Safety Pharmacology Core Battery." It was also agreed that such studies should ordinarily be conducted in compliance

with principles of GLP and only general study designs were described. The EWG wished to limit the scope of the core battery exclusively to the three critical organ systems for the reason described above, but as safety pharmacology was originally envisioned in the early draft of the ICH S6 guideline (Anon 2012a, b), the study of the renal system had also been described. The request to study renal function before FIM continues to be part of ICH S6 despite its revision in 2009, but in practice, this functional test is not asked for at that early time of development by regulators, except if there is concern.

At the meeting in Brussels, consensus of the members was also achieved that "follow-up studies" of the "core battery" would be conducted to provide a greater depth of understanding of the pharmacodynamic properties of the molecular entity than that provided by the standard designs of the core battery studies. There was also agreement that the follow-up studies would be uniquely designed to test specific hypotheses. Although not comprehensive, a list of examples of different types of follow-up studies were cited in the guidelines. The EWG also devised another category of studies, the "supplemental" study, which were carried out when evaluation of other organ systems (e.g., renal/urinary system, autonomic nervous system, gastrointestinal system, etc.) was required. The EWG agreed that the "follow-up" and "supplemental" studies should be conducted in compliance with GLP to the greatest extent feasible and that at minimum having sufficient documentation to assure being able to reconstruct the study would be of greatest importance.

In addition to the categorizations described above, two other categories of pharmacodynamic studies were described in the ICH S7A guidelines at the request of ICH M4S EWG (Anon 2001a, d). These included the primary pharmacodynamic and secondary pharmacodynamic studies, which were described in order to distinguish the requirement for GLP compliance for safety pharmacology studies, but not for primary or secondary pharmacodynamic studies.

2.3 General Considerations on In Vivo Studies

In conducting in vivo studies, it is preferable to use unrestrained, unanesthetized animals that are conditioned to the laboratory environment, always paying attention to the welfare of animals. In the discussions of the use of unanesthetized animals, the avoidance of discomfort or pain was considered of foremost importance. The EWG said that in well-characterized in vivo test systems, the repeated study of positive control agents may not be necessary. The latter is indicative of the animal welfare practice of the 3Rs (reduction, refinement, and replacement (Holmes et al. 2010). With regard to biotechnology-derived products that achieved high specific receptor targeting that has been demonstrated in an appropriate animal species, the EWG made a definitive statement that it is often sufficient to evaluate safety pharmacology endpoints as a part of toxicology and/or pharmacodynamic studies (provided that exposure data are available in the latter). As a result, with such strategy separate safety pharmacology core battery studies need not be

conducted. This principle is considered to be one of the reasons for a recent trend toward combining safety pharmacology endpoints into toxicology studies (Redfern et al. 2013; Vargas et al. 2013). Altogether safety pharmacology should not be considered as a stand-alone discipline. Close cooperation among safety pharmacology, pharmacokinetics, and toxicology can facilitate the overall development of a new molecule. Like all safety studies, safety pharmacology needs to be supported with drug pharmacokinetic information, but that could, for example, be derived from toxicology studies. The combined knowledge from these disciplines can optimize the calculation of safety margins (as outlined by Redfern et al. 2003). Another example is the selection of the high dose in safety pharmacology studies; here toxicity data can help to justify the limit of the top dose selected.

However, upon reflection by the safety pharmacology community over the past almost 15 years, the view that safety pharmacology endpoints can be incorporated into toxicology studies has been challenged, particularly in the case of cardiovascular measurements. Scientists have recognized that the level of precision of cardiovascular safety pharmacology endpoints collected in dedicated safety pharmacology studies could not be reproduced without careful attention to the study conditions in definitive toxicology studies (Guth et al. 2009; Leishman et al. 2012; Pettit et al. 2009; Redfern et al. 2013). This awareness has led vendors to develop technologies that can be adapted to toxicology studies in order to mitigate the imprecision of many of the standard methods that existed at that time. Included are systems to evaluate cardiovascular and respiratory function, e.g., electrocardiogram (ECG), blood pressure, and respiratory rate and volume using jacketed technologies; see reviews from Authier et al. (2013) and Redfern et al. (2013). In addition, a similar concern has prompted organizations to introduce dedicated trained staff capable of studying CNS function in the course of subchronic and chronic toxicity studies. Together, this heightened sensitivity to the quality of data used in the decision making and emergence of technical and scientific capabilities has enhanced the confidence in the critical data from toxicology studies that are used to assess the pharmacodynamic risk posed by intermediate- to long-term exposure to small molecules and biologics.

Cardiovascular telemetry, which was strongly recommended by the FDA for in vivo studies, was a relatively new technology at that time of the ICH S7 deliberations. The introduction of the telemetry systems facilitated the conduct of in vivo studies in unrestrained, unanesthetized animals acclimated to the experimental conditions, enabling evaluation of the standard cardiovascular core battery endpoints (e.g., blood pressure, heart rate, and ECG) and allowing the reutilization of animals in subsequent studies. Recognizing the significant advantages offered by this technology, it was strongly embraced by the EWG members as a revolutionary advancement in the conduct of cardiovascular safety studies. Here was a prima facie example of regulation embracement of a new technology that preceded widespread acceptance and incorporation within divisions/laboratories conducting these studies. One author (LK) recalls receiving several communications from international scientists conducting cardiovascular safety pharmacology studies at this time to inquire whether telemetry technology would be acceptable in support of regulatory dossiers.

2.4 Achievement of Step 4 of ICH S7A and Initiating ICH S7B as a New Topic (The Sixth San Diego EWG Meeting in November 2000)

The first US president of the twenty-first century was supposed to be elected on November 7, 2000, the day before the last day of the San Diego meeting of the ICH S7A EWG (November 5–8, 2000), but the outcome of that contentious and closely watched election was not decided until 5 weeks later by the United States Supreme Court. The Step 4 document with regulatory authority members of S7 EWG signatures' affixed was submitted to the ICH Steering Committee on November 8, 2000, with the request for adoption. However, the S7 EWG could not come to consensus on a list of suitable test systems and assays for prediction of the ECG anomalies associated with QT prolongation, such as torsades des pointes and sudden death, that had been extensively documented over the preceding 10 years (Bass et al. 2008, 2011). The difficulty to come to consensus was likely due to the state of the science in this field and the dearth of data that would inform on best practices, as well as the lack of predictive biomarkers of proarrhythmia that existed at that time. As a result, this issue was carried over to a new ICH topic which allowed the ICH Steering Committee to approve the Step 4 ICH S7 document as it existed at the time, "S7: Safety Pharmacology Studies for Human Pharmaceuticals," but designated ICH S7A rather than S7 in order to continue discussions on the ECG topic by a newly sanctioned EWG. This new topic was designated ICH S7B.

As a result of this decision, the S7A EWG members left the ICH Steering Committee meeting room and added the following Note 3 to the end of the S7A Document to encourage the industry to submit data to the regulatory authorities that would inform on the study of ECGs, assessing proarrhythmic risk.

Note 3. A guideline (S7B) will be prepared to present some currently available methods and discuss their advantages and disadvantages. Submission of data to regulatory authorities to support the use of these methods is encouraged.

At that point, having met the goals of ICH to achieve Step 4 guidance on the practices of safety pharmacology, the S7A EWG was formally disbanded.

2.5 S7A and S7B EWG and Cultural Bonding

On the first day of the first meeting in Brussels, all the members of Safety EWGs were invited by the Safety EWG Coordinator of European Federation of the Pharmaceutical Industry Association (EFPIA) to a gathering that included wine tasting. All the members of S7 EWG joined the party which was held in the coordinator's family home. All of his family, including his parents, brother, and sister-in-law, were welcoming to this collection of scientists from around the world. Nearly 50 EWG members enjoyed splendid wine and delicious home cooking of a Brussels family, which allowed a deepening of mutual friendship and trust that would foster the ability to carry out frank exchanges of serious opposing opinions regarding different agenda topics over the course of several years. The Japanese members who joined the party were only those of the ICH S7 EWG. As a result of

the comradery created by this event, a gathering of Safety Party members would become a routine event adjoined each meeting of the ICH S7A EWG, and what would become the ICH S7B EWG. Individuals would bring wines from their respective countries and share many details about their selections. For example, the Japanese members would bring Sake to these gatherings and share the background to its production and the important relationship of their selection to Japanese culture. Dr. Joseph DeGeorge of FDA and Dr. Peter Grosser of Health Canada kindly organized each gathering, and all members took part in this important activity of fostering the relationships that would carry each of the members through the important and at times contentious deliberations of creating harmonized guidances to a successful conclusion.

3 S7B: The Nonclinical Evaluation of the Potential for Delayed Ventricular Repolarization (QT Interval Prolongation) by Human Pharmaceuticals (2000–2005)

The membership of the ICH S7B Expert Working Group and a chronicle of the timelines and milestones leading to adoption are presented in Tables 3 and 4, respectively.

Table 3 ICH-S7B Expert Working Group members

Party	Experts	
MHLW	Kannosuke Fujimori (PMDA)[a, b] Tsuyoshi Ando (PMDA)	Kenichi Nakazawa (NIHS)
JPMA	Munehiro Hashimoto (Pfizer)[c, b] Keiji Yamamoto (Takeda)	Toshiyasu Hombo (Astellas)[b]
EU	Klaus Olejniczak (BfArM)[b]	
EFPIA	Gerd Bode (Altana Pharma)[b] Tim Hammond (AstraZeneca)	Andrew Sullivan (GSK)[b]
FDA	Joseph DeGeorge (CDER)[b] David Morse (CDER)	John Koerner (CDER) David Kram (CDER)
PhRMA	James Green (Biogen)	Peter Siegl (Merck)
EFTA	Beat Schmid (Swissmedic)	
Canada	Colette Strnad (Health Canada)	Peter Grosser (Health Canada)[b]

[a]Rapporteur from Step 2 through Step 4 sign-off
[b]S7A&B-EWG
[c]Rapporteur from Step 0 though Step 2 sign-off
Members *MHLW* Japanese Ministry of Health, Labour and Welfare, *JPMA* Japanese Pharmaceutical Manufacturers Association; *EU* European Union, *EFPIA* European Federation of the Pharmaceutical Industry Association, *FDA* United States Food and Drug Administration, *PhRMA* Pharmaceutical Research and Manufacturers of America, *EFTA* European Free Trade Association, *PMDA* Pharmaceutical Medical Devices Agency, *NIHS* Japanese National Institute of Health Sciences, *BfArM* German Federal Institute for Drugs and Medical Devices, *GSK* GlaxoSmithKline, *CDER* Center for Drug Evaluation and Research, *Swissmedic* Swiss Agency for Therapeutic Products

Table 4 Chronology of S7B Expert Working Group (EWG) meetings

EWG	Date	Place	Notes	Step
First	May 2001	Tokyo		1
Second	August 2001	Ottawa	Extra	1
Third	October 2001	Toronto	Extra	1
Fourth	February 2002	Brussels		2
Fifth	September 2002	Washington, DC		3
Sixth	February 2003	Tokyo	Start of E14	3
Seventh	July 2003	Brussels		3
Eighth	November 2003	Osaka		3
Ninth	June 2004	Washington, DC	Step 2 of E14	Revised Step 2
Tenth	May 2005	Brussels	Step 4 of E14	Step 4

Note: Extra refers to two meetings held by the ICH S7B Expert Working Group that were outside of the regulatory scheduled meetings of the ICH Steering Committee. Step: reader is referred to the ICH website for a definition of the ICH Process (http://www.ich.org)
Note: Achieved Step 5 of S7B and E14 in JAPAN Oct. 23, 2009, 4 years following adoption of these guidelines in EU and USA

Note: Delayed by 3 years, but in parallel with the timing of ICH S7B, clinical guideline on study of drug-induced delay in cardiac ventricular repolarization, ICH E14: The Clinical Evaluation of QT/QTc Prolongation and Proarrhythmic Potential for Non-antiarrhythmic Drugs, were written and adopted (2003–2005).

3.1 Early Events Associated with ICH S7B: Step 1 to Step 2 (May 2001–February 2002)

Just prior to the first EWG meeting in Tokyo in March 2001, a guidance document entitled an "Assessment of the QT prolongation potential of nonantiarrhythmic drugs" was issued by Health Canada's Therapeutic Products Directorate (TDP) without advance notice to the worldwide community (http://www.hc-sc.gc.ca/hpb-dgps/therapeut/htmleng/guidmain.html). This document specified the conditions for nonclinical (safety pharmacology) and clinical studies intended to evaluate the proarrhythmic potential of drugs, providing a full-scale guidance covering the widest range of recommendations for the investigation of test article effects on cardiac ventricular repolarization since the publication of the regulatory guidance, the "Points to Consider, The Assessment of the Potential for QT Interval Prolongation by Non-cardiovascular Medicinal Products," by the EMEA (CPMP) in December 1997 (Anon 1997a). The issuance of this comprehensive guidance had the effect of elevating the unofficial role that would be served by Health Canada on the ICH S7B EWG, which up to this point had been an "observer" during the deliberations of ICH S7A. Their representatives would now become more vocal contributors to the deliberations, most often aligning themselves with the FDA representatives during the debates. In addition, the Health Canada would host two extraordinary meetings for S7B-EWG members in Ottawa in August 2001 and again in Toronto 2 months later in October 2001.

A third extraordinary meeting of the S7B-EWG in Toronto in October 2001 was convened in place of the scheduled EWG meeting in Brussels, which had been canceled due to the tragic terrorist attack on the USA on the September 11, 2001. The US FDA parties were not allowed to fly for security reasons, but to assure that progress on the guidelines, a site was selected in a neutral country to which EU and Japanese ICH members could fly and the US members could drive. The third extraordinary meeting was carried out with the utmost care and attention to the security of the S7B-EWG members in the aftermath of September 11. For example, the venue of the meeting was a secure resort managed by a private winery 90 and 30 km, respectively, from cities of Toronto and Niagara. The EU and Japanese members of EWG were emotionally and physically exhausted by the stressful flight to North America under severe security conditions at the airports. The US members arrived at the location of the meeting, having driven a long distance from their homes to the meeting place in Canada. This was a very difficult time for the international community, which was not prepared for the events of September 11, 2001, nor the life that our societies would eventually learn to live in the aftermath of this tragic event and those events around the world that were to follow in the coming years.

The draft S7B guidance reached Step 2 (signed off by all topic leaders of the EWG and transition of rapporteur from the pharmaceutical industry member from the JPMA to the regulatory member from the MHLW) at the fourth meeting in Brussels in February 2002, after four face-to-face meetings plus a videoconference of the S7B EWG, all occurring within a year of adoption of the topic by the ICH Steering Committee.

The most significant and valuable outcome of the fourth meeting in Brussels was the establishment by the ICH Steering Committee of a parallel topic of creating clinical guidelines for the evaluation of new agents for the potential to prolong QT and induce proarrhythmia, ICH Topic E14. The ICH Steering Committee also selected ICH E14 EWG, who would deliberate over the next 3 years developing a clinical guidance document.

On this occasion, the FDA and Health Canada laid out the following requirements to avoid delaying the preparation of the clinical guidance document. They requested:

1. The document will be fast-tracked by eliminating the standard ICH procedures (preparation of the concept paper) and by also inviting experts outside the ICH process to the workshops.
2. If the ICH process results in falling behind the established timeline, the FDA and Health Canada reserve the right to withdraw the document from the ICH process and prepare the document independently.

The ICH Steering Committee accepted the requirements of the FDA and Health Canada and established ICH Topic E14. They also agreed to the “fast-track” procedure. Interestingly, according to the order of ICH-E topics, this topic would have actually been Topic E13. Although, while not understood by all members of the international community of why designation E13 would be a concern, it was

agreed unanimously by the Steering Committee to retire E13 and assign E14 to this topic.

An additional note at the meeting in Brussels, Dr. Joseph DeGeorge, who played a leading part in discussions at EWG meetings from the beginning of ICH S7A to the accomplishment of ICH S7B Step 2, made the Brussels meeting his final event as a representative from the FDA, as he was going to retire from the agency. A celebration was held in his honor by the S7B-EWG and other EWG members. Over 100 ICH members gathered to express their appreciation for his contributions to ICH and wish him well in his new career.

3.2 Events Associated with ICH S7B (Transition from Step 3 to a Revision of Step 2) (February 2002–June 2004)

To reach Step 4, it was considered necessary to compile datasets from preclinical safety pharmacology assays of cardiac repolarization recommended in the Step 2 draft guidance (Bode and Olejniczak 2002; Hashimoto 2003; Fig. 2) in order to verify the reliance of these assays to predict clinical QT prolongation. In support of this work, prospective preclinical studies were carried in different collaborations across various institutions and organizations in the EU, Japan, and USA with the goal of building the databases that would inform the EWG. The ICH S7B-EWG meeting waited for these results before proceeding.

Among all of the work in progress at the time, two major collaborative projects were at the center of this review, one under the auspices of the International Life Sciences Institute, Health, and Environmental Sciences Institute (ILSI-HESI), Cardiovascular Safety Subcommittee Initiative with membership from Academia,

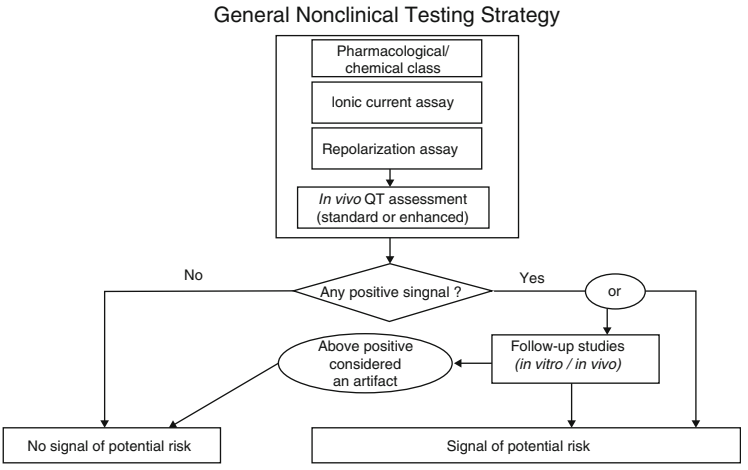

Fig. 2 An early version of the ICH S7B testing strategy, Step 2 circulated for comment included an evaluation of the action potential duration (repolarization assay) as a primary test with regard to assessing risk. This assay was moved to a secondary role by the time of the Step 2 revision to the draft guidance document

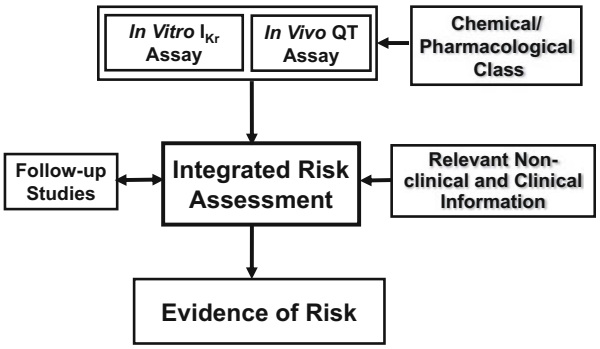

Fig. 3 Final ICH S7B testing strategy as it appears in the guidance, ICH S7B

PhRMA, EFPIA, and FDA and the other, the QT Interval Prolongation: Project for Database Construction (QT PRODACT), under the direction of the Japanese Pharmaceutical Manufacturers Association and the Japan Association of Contract Laboratories for Safety Evaluation (JACL). The FDA also had a separate effort to continue to assess the clinical and preclinical databases from applications for approval of new drugs (New Drug Applications, NDAs) for the level of concordance. The investigational results of ILSI-HESI and QT-PRODACT were first presented at the Safety Pharmacology Society Annual Meeting in Amsterdam held in September 2003 and subsequently appeared in publications (Hanson et al. 2006; Omata et al. 2005). In addition, the results of a retrospective literature survey carried out by the Association of the British Pharmaceutical Industry (ABPI) were also reported at the S7B-EWG meeting and in the publication from Redfern et al. (2003). Collectively, these data allowed the ICH S7B EWG to focus the strategy on two assays, the in vitro assessment of human Ether-a-go-go-Related Gene (hERG) channel activity and in vivo in an animal model of QT prolongation (Fig. 3). Assays of action potential duration (APD), which were part of the primary decision tree in the Step 2 document (Bode and Olejniczak 2002; Hashimoto 2003) (Fig. 2), were shown to be suboptimal in predicting QT prolongation in humans (Hanson et al. 2006; Omata et al. 2005). The explanation for this observation was that the effects of test compounds on the APD assays were the result of summation of the collective ion channel properties of a small molecule, which may in some cases have opposing effects, resulting in the absence of a change in the APD. As a result, the recommendation made in the guidelines was that the APD assay serve as a "follow-up" study to better understand the mechanism of QT prolongation and no longer as a primary assay for decision making (Fig. 3). As an additional by-product of the success of the QT-Prodact collaboration and the relationships built in Japan, was establishing the Japanese Safety Pharmacology Society (JSPS) in December 2009 (Bass et al. 2011); the reader is also referred to the JSPS website http://www.j-sps.org/sub1.html for more information.

With regard to ICH E14, the first EWG meeting was held in Tokyo in February 2003. Thereafter, a joint meeting of the ICH S7B and ICH E14 members was held at each of the EWG meetings in order to maintain consistency between the two draft guidance documents. To that end, both ICH S7B and ICH E14 EWGs were directed

by the ICH Steering Committee to achieve Step 4 at the same time. As a result of this action, the S7B draft guidance awaited the completion of E14 draft guidance for another 2 years before arriving at Step 4.

In June 2004, the E14 draft guidance document reached Step 2 in the EWG meeting held in Washington, DC. The S7B document also reached a revised version of Step 2 after revisions were made to align the draft S7B with the draft E14. The process of revising a Step 2 document after signatures had been affixed was an unprecedented step in ICH. At this time in history, the state funeral for President Ronald Reagan, the 40th president of the USA, was held just prior to the EWG meeting in Washington, DC.

Upon achieving Step 2 of ICH E14, there was strong disagreement between the FDA and the allied EU (EMEA) and Japanese (MHLW) members of whether the nonclinical data collected in safety pharmacology according to ICH S7B would predict the outcome of the clinical evaluation of QT prolongation. The FDA members were skeptical of clinical predictability of the nonclinical data, whereas the EU and Japanese members advocated that preclinical assays provided signals which could be predictive. The FDA members directed that the "thorough QT studies" be conducted in "almost all cases" regardless of the results of nonclinical assays. The EU and MHLW members considered that the "thorough QT studies" could be excluded if there was sufficient evidence from nonclinical assays to suggest a low level of risk. This difference of opinion between the regulatory members was never bridged and so a compromise was established to accept regional differences in the implementation of ICH E14. From the FDA to the safety pharmacologists to completely exclude the possibility of QT prolongation in humans based on preclinical data was going to be impossible to meet. It was emphasized that the objective of all preclinical safety studies is not exclusion, but rather information on the probability of possible risks. Nevertheless, the following is the excerpt from E14 Step 2 document: "At present, whether or not the non-clinical testing can exclude risk for QT/QTc prolongation is controversial. Conduct of the "thorough QT/QTc studies" as described in Sect. 2.1.2, would be needed in almost all cases in the regions where non-clinical data are not considered to be able to preclude risks of QT/QTc prolongation. In the regions where non-clinical data are considered informative enough about the risk of QT/QTc prolongation in humans, the recommendations in this guidance for the clinical evaluation of QT/QTc could be modified." The reader should note that this provocative language in the Step 2 document did not survive to the final Step 4 version.

The conflicts concerning reference to clinical predictivity of nonclinical data in the ICH E14 Step 2 document were a significant challenge for the S7B EWG. In addition, a difference of opinion among the S7B members also existed as to which chemical classes would require study according to ICH S7B, when would the studies be required relative to the stages of the clinical program, which studies were considered essential, and which were to be carried out in compliance with GLPs. All of these questions remained to be resolved over the course of the several face-to-face meetings leading up to Step 4. In brief, Table 5 details the key differences in opinion which existed between the regulatory members of the S7B and E14 EWGs and the final recommendations which resulted in achieving Step 4.

Table 5 Regional difference between EU/MHLW and FDA regarding the draft ICH S7B guidance that allowed transition from Step 2 to Step 4

	Step		
	(Revised) Step 2		Step 4 (resolution in final guidance)
Issue	EU + MHLW	FDA	
Clinical predictability of ICH S7B-related findings	Predictable	Questionable whether Predictable?	Predictable (for the moment)
When is ICH S7B necessary?	Almost all cases	Case by case	Almost all cases
Timing of ICH S7B	Prior to first administration in humans	Prior to TQT study or NDA	Prior to first administration in humans
Primary study of S7B	CV Telemetry + hERG + APD	CV Telemetry + hERG	CV Telemetry + hERG
Necessity of GLP compliance	Essential	Questionable whether needed?	Essential
Necessity of TQT study	Case by case	Almost all cases	Case by case (for the moment)

EU: European Union, *MHLW*: Ministry of Health Labor and Welfare, *FDA*: Food and Drug Administration, *TQT*: Clinical Thorough QT study, *NDA*: new drug application, CV Telemetry: cardiovascular telemetry studies, hERG: human ether-a-go-go gene, *APD*: action potential assay

3.3 Events Leading to Step 4 of ICH S7B (June 2004–May 2005)

In the EWG meeting in Brussels in May 2005, the ICH E14 document was advanced to Step 4 as a result of the FDA agreeing to delete the description of "regardless of the results of non-clinical studies, a thorough QT/QTc study should be conducted in almost all cases" and "acceptance of regional differences in the operation of the Documents." As a result of this decision, both ICH S7B and ICH E14 were able to progress to Step 4. In the case of ICH S7B, the FDA accepted many of the positions argued by the EMEA and MHLW concerning which chemical classes would require study, the required timing of studies relative to the stages of the clinical program, which preclinical studies were considered essential, and which were conducted in compliance with GLPs.

Final Note

Dr. Munehiro Hashimoto, who served as the pharmaceutical industry-rapporteur at the EWG meetings of ICH S7A and ICH S7B passed away on September 23, 2005, approximately 4 months after ICH S7B had reached Step 4. Those of us from around the world who had worked with him sincerely pay our respects to his wife, Keiko Hashimoto, and their two sons, Yusuke and Keisuke. We acknowledge, as his comrades and colleagues, the tireless contributions that he made to the field of safety pharmacology (Nomura et al. 2006). In honor of his accomplishments, the Safety Pharmacology Society invited Dr. Hashimoto's bereaved family to the Sixth

Annual Meeting held in San Diego, California, in September 2006. A ceremony was conducted commemorating his accomplishments in the field of safety pharmacology and significant contributions to successful adoption of the ICH S7A and ICH S7B guidances.

4 The Period That Followed Adoption of ICH S7A and ICH S7B (2001 to Present)

The period that followed adoption of ICH S7A since 2001 saw the implementation of laboratories fully capable of complying with the guidance, with some institutions choosing to outsource some, or all, of their GLP studies rather than committing in-house resources to that effort, in particular, establishing laboratories compliant with 21CFR Part 11 (required by the FDA) and GLPs (Anon 2004b, 2000a, b). This move to outsource studies by some institutions was a foreshadowing of events to come in the latter part of 2000, that would be brought about by the economic collapse of worldwide economy leading to a significant and long-lived recession. The impact of the economic collapse and other external pressures being placed on the pharmaceutical industry and field of safety pharmacology will be further detailed later in this chapter.

Not all institutions chose the path of working through contract research organizations (CROs) in order to conform to the regulatory requirements for safety pharmacology. Instead, many companies chose to align their standard assay procedures according to 21CFR Part 11, allowing electronic data collection in the course of carrying out GLP studies (Anon 2000a). As a result of this action, these laboratories were beginning to standardize the design of studies and the format for preclinical pharmacodynamic data collection, which up to that time had been tailored to address specific program issues and hypotheses and varied based on the requirements of a study.

As an extension of uniformed data collection, the FDA launched a warehousing initiative to require sponsors to provide raw data from preclinical safety studies, including data from safety pharmacology studies, in a standard format to allow for further analysis by the agency (Wood and Kramer 2011). In this effort, they recruited software vendors and members of the pharmaceutical industry to work out how to standardize data formats that would facilitate compliance with the program goals. The program which is in progress at this time was given the acronym SEND (Standard for Exchange of Nonclinical Data), and the reader is referred to the following reference for more information (Wood and Kramer 2011).

Implementation of ICH S7B, which achieved Step 4 in May 2005, was completed in the EU and USA in the same year. However, in Japan, implementation was delayed until 2009. In a society that had great concerns about putting healthy individuals at risk for toxicity from a pharmaceutical, where there was no intention to provide clinical benefit, the requirement in the Step 4 ICH E14 guidance for the use of a positive control agent, e.g., moxifloxacin, in the Clinical Thorough QT (TQT) study, posed a significant dilemma for the MHLW. This issue would need to be reconciled before adoption of the guidance could occur in Japan. Since the

progress in developing the ICH E14 guidance was conjoined with the ICH S7B guidance by the ICH Steering Committee at the time that the clinical initiative was adopted, the delay in ICH E14 in Japan meant that ICH S7B would also be delayed until such time that a solution to the question of the need for a positive control agent in the Phase I TQT clinical trial was found.

That resolution came when the ICH Steering Committee adopted a process to respond to questions from the pharmaceutical and regulatory communities by sanctioning an ICH E14 Implementation Working Group (IWG) to serve as an advisory panel. As a result of establishing this working group, ICH E14 achieved Step 4 in 2005, which allowed the first Questions and Answers (Q&As) session of the E14 IWG in June 2008. This working group was able to respond to the question posed by the Japanese regulators by clarifying the expectations of the positive control agent for the TQT study. Having final resolution to this important concern, both ICH E14 and ICH S7B were adopted in Japan in 2009.

Since the implementation of ICH E14 worldwide, the guideline has also been fraught with challenges and questions about the specific methodology being prescribed, as well as a reevaluation of new approaches to the clinical study design based on emerging data. This process of Q&A was the intention in establishing the IWG by the ICH Steering Committee in 2005. The first response to Q&As was reported in 2008 and a second in 2012. The reader is referred to the following link for further details of these exchanges (http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Efficacy/E14/E14_QAs_R2_Step4.pdf).

Importantly, during the second Q&A session in 2012, there was also a review of preclinical data which had emerged since the adoption of the ICH S7B guidance in 2005. The purpose of this review was to decide whether these new data would have any impact on the existing preclinical guidelines. The outcome of that meeting was that there wasn't sufficient new data to recommend any changes to the ICH S7B guidance at that time. However, as will be noted in the following section, there are a number of initiatives in progress that may have an impact on ICH S7B, in particular a new FDA challenge that is being championed by several different consortia working collaboratively to develop an integrated algorithm of ion channel assays and in silico models, as well as the innovative inducible pluripotent stem cell cardiomyocyte platform to predict proarrhythmic risk (Cavero and Holzgrefe 2014; Chi 2013; Sager et al. 2014; Sager and Kowey 2014).

The conclusion of the IWG at their meeting in 2012 was that they recognized that there were a number of nonclinical initiatives underway by various consortia of academics and regulatory and pharmaceutical industry bodies. These included the HESI, BfArM European Concordance Project, Association of the British Pharmaceutical Industry (ABPI)—Animal Model Framework, TI-Pharma PK/PD Assessment, the European Centre for the Validation of Alternative Methods (ECVAM), and Cardiac Safety Research Consortium (CSRC), all in general raising the question regarding the level of concordance between the results from preclinical assays of cardiac ventricular repolarization and the outcome of clinical studies of QT prolongation (Chain et al. 2013; Piccini et al. 2009; Pierson et al. 2013; Sarganas et al. 2014; Stummann et al. 2009; Trepakova et al. 2009; Valentin et al. 2009).

Among these initiatives, the project of the HESI Cardiovascular Safety Subcommittee represented an analysis of the largest database among the different consortia, a survey of 150 molecules studied in the clinic for QT prolongation (Koerner et al. 2013). This initiative is comparing the concordance between preclinical cardiac repolarization assays and the results from the "TQT" study; data were collected from INDs and NDAs submitted to the FDA between the periods of March 2006 and July 2012. Due to the proprietary nature of these datasets, the FDA populated the spreadsheets with the data and carried out a quality control and statistical analysis based on a methodology that was devised by the subcommittee in advance of the collection of data. The summary results of this analysis of 150 compounds has been presented at conferences and is due for publication in 2015 (Koerner et al. 2013).

Another consortium initiative worth highlighting brings together the FDA, HESI, and CSRC. In a recent publication, this workgroup challenged the scientific and regulatory community to demonstrate that integrating the results of a panel of assays will predict proarrhythmic risk. This initiative has been entitled "Comprehensive In vitro Proarrhythmia Assay" or CiPA (Cavero and Holzgrefe 2014; Chi 2013; Sager et al. 2014; Sager and Kowey 2014), and the goals are to demonstrate that an integrated algorithm can be devised that is sufficiently predictive of proarrhythmia that the scientific and regulatory communities can abandon ICH E14 by July 2015 and revise the ICH S7B guidance to include this testing strategy by July 2016 (Cavero and Holzgrefe 2014; Chi 2013; Sager et al. 2014; Sager and Kowey 2014). Given the progress of this working group to date, achieving the milestones originally set out for 2015 and 2016 are highly in doubt. For example, the current framework for the study paradigm relies on a yet to be evaluated algorithm which combines study results from the test of small-molecule agents in an assortment of critical human cardiac ion channel assays, integration of these data using in silico models of cardiac proarrhythmia, and the use of iPSC cardiomyocytes to complete a comprehensive review of a compound's risk for proarrhythmia (Cavero and Holzgrefe 2014; Sager et al. 2014). Together these data are intended to broadly predict the proarrhythmic risk, not only of QT prolongation, but cardiac arrhythmias such as torsades des pointes. If successful, the CiPA initiative would allow sponsors and regulators to judge the risks associated with advancing new molecules into development, obviating the need for a mandatory "TQT" study, a move that would significantly contribute to reducing the time and expense of drug development.

Since the adoption of the ICH guidances, advancements in the practice of safety pharmacology not only focused on complying with international regulations but also to evolve and align with the scientific and technological discoveries that were facing this young field. As examples, advances in our understanding of molecular biology of novel new targets, the promises offered by inducible pluripotent and embryonic stem cells in predicting human toxicities, and the ability to map the human genome have brought about not only an understanding of potential targets of disease modification, but also the potential for those which may lead to unwanted adverse pharmacodynamic events (AEs). In other words, these scientific advancements offered the safety pharmacologist the chance to assess

pharmacodynamic toxicity in human tissues or genetically modified animal models. These discoveries also offered a way of probing for theoretical concerns associated with novel pharmacodynamic targets once molecular, small-molecule, and biologic tools became available that could be used to modulate the target.

The advances of new technologies, concerns of regulators and the public for safe new effective therapies for unmet medical needs, and the pressures on our industry to respond to the economic and practical challenges of discovering and developing new therapies have led to a reassessment of strategies to progress compounds to the marketplace in safe, cost-effective, and expeditious ways. For many years, the strategy for solving the high rate of compound attribution was to increase the number of molecular agents advancing into development, so-called shots on goal. However, this approach carried out over many years was a complete and expensive failure costing the industry billions of dollars (Cook et al. 2014; Hay et al. 2014; Holdren et al. 2012; Munos 2009; Urban et al. 2014). The belief today is that if translational biomarkers of both safety and efficacy can be effectively used in early clinical development to identify those molecules with the best opportunity to progress safely through the clinical phases, demonstrate proof of biology and ultimately efficacy in the targeted patient populations, that this approach may be successful in mitigating the risk of a compounds failing in the latter stages of development before a large commitment of resources and time have been made (Bass et al. 2009; Cook et al. 2014; Holdren et al. 2012; Munos 2009).

Identifying the most promising molecules to advance into development has led to a new mandate for safety pharmacology. More and more programs in safety pharmacology that had been established in development were now migrating into discovery to identify mechanism-based liabilities associated with novel targets in early discovery, demonstrating concerns regarding the pharmacodynamic toxicities of core chemical structures in the lead finding phase and assuring that there were no significant unwanted properties present in the final development candidate(s) in the lead optimization phase. No longer simply focusing on regulatory GLP studies, safety pharmacologists were now redirecting their resources to early screening and investigational studies to select the best pharmaceutical targets and candidates to advance into later discovery and development phases (Bass et al. 2009; Cook et al. 2014). Whether the new strategies devised to mitigate compound attrition are successful will be judged by the number of new registrations achieved over the next 5–15 years (Cook et al. 2014).

Under the pressures of the economic environment that has enveloped the world in the first decade of the new millennium, the results have included contraction in the size of pharmaceutical institutions, fewer internal personnel and resources to carry out the job of discovering and developing new drugs, and a more focused commitment of those resources to a smaller portfolio and fewer therapeutic areas of research (Kaitin and DiMasi 2011; Munos 2009; Munos and Chin 2009; Urban et al. 2014). Organizations are moving routine regulatory assays to other centers around the world, such as contract research organizations (CROs), as a strategic change to leverage the deployment of internal resources to high-priority projects and activities and to manage costs. In addition, many academic institutions are

remodeling themselves to provide scientific services to and partner with pharmaceutical industry, including carrying out studies that fall under the umbrella of safety pharmacology. As a result of these changes, some of our safety pharmacology colleagues are similarly moving to the contract and academic sites where their new employment opportunities exist.

5 Vision of the Future of Safety Pharmacology, Beyond the Present

The forces that have shaped safety pharmacology to its current embodiment as described in this chapter can provide clues as where to look for the forces that will continue to shape the discipline in future years. The evolution of safety pharmacology is a result of past traditions of pharmacologic bioassay-based drug discovery, general pharmacology profiling of new drugs for both additional useful pharmacological activities and potential deleterious effects on critical organ systems, and incorporation of advances in science and technology that enhance potential cross-species translation of experimental endpoints. These were the underpinnings for the ICH S7A and S7B EWGs and the emerging Guidance for Safety Pharmacology in the early to mid-2000s, and as there has been no revisions to either ICH S7A or ICH S7B since that time, the ICH Safety Pharmacology guidances are unchanged. However, as there is growing recognition and movement of safety pharmacology into the drug discovery space, this will dictate change and have the potential to influence the ICH guidances in the future (Bass et al. 2009; Cavero and Holzgrefe 2014; Chi 2013; Curtis and Pugsley 2012; Ewart et al. 2013; Laverty et al. 2011; Pugsley et al. 2013; Pugsley and Curtis 2012; Redfern et al. 2013; Redfern and Valentin 2011; Sager et al. 2014).

The academic training programs of the 1970s and early 1980s produced the "pinnacle" generation of research scientists trained in advanced sophisticated intact animal research techniques that powered the development and advancement of the Safety Pharmacology Core Battery and ancillary bioassays over the past 15 years and were a factor for inclusion of specific language in ICH S7A. Section 2.3.2 describes "In conducting in vivo studies, it is preferable to use unanesthetized animals. Data from unrestrained animals that are chronically instrumented for telemetry, … are preferable to data from restrained or unconditioned animals." However, the numbers of such so-trained young scientists, and indeed those having any interest in animal research, have greatly diminished in recent decades—raising legitimate questions as to from where the next generation of safety pharmacologists will arise? A significant challenge to the field, attracting, training, and certifying investigators in integrative approaches to physiology, pharmacology, and toxicology in order to ensure the future of the discipline is a significant priority (Bass et al. 2011; Collis 2006; Valentin and Hammond 2008). The availability of trained safety pharmacologists to address these concerns is quickly disappearing as these individuals are approaching retirement. The training of new safety pharmacologists was recognized over a decade ago as a critical need for our discipline that was being

underserved in curriculum being offered by academic institutions around the world. The paucity of training in integrative biomedical sciences at academic institutions has had detrimental long-lasting effects such as an impact on (1) the development of in vivo animal models of human function and disease, (2) the skills required to conceptualize biomedical hypotheses and experiments that will leverage knowledge of the intact animal physiology, (3) the process of nonclinical and clinical drug discovery and development study, and (4) the ability to integrate complex data sets in order to formulate an integrated risk assessment. It is reassuring, however, to see the emergence of training programs in the EU, Japan, and USA on drug safety and safety pharmacology more specifically (e.g., Valentin and Price 2007); the reader is also referred to the JSPS website http://www.j-sps.org/sub1.html; some of those programs include accreditation of institutions and individual scientists. More recently, formal Certification in Safety Pharmacology that allowed granting of the title, Diplomate in Safety Pharmacology (DSP), was implemented by the Safety Pharmacology Society. Formal certification is intended to recognize individual's didactic and practical training in science and practice of safety pharmacology (http://safetypharmacology.org). The development of programs designed to identify and help train the "next generation" of safety pharmacologists that will lead this critical field in the future is of paramount importance. To be successful and productive, any initiative will require adequate public and private financial support.

In North America and EU, animal research is facing social and political resistance as never seen before, as (unfairly) an archaic and unnecessary "relic" of science past. Indeed, the combination of lack of trained scientists and negative public pressure is already shifting conduct on nonclinical regulatory animal bioassays to other regions of the world, where animal use is more acceptable (Chapman et al. 2013; Mangipudy et al. 2014).

Finally, the pharmaceutical industry has enjoyed a "golden age" for the past 50 years in terms of products and profitability; however, worldwide pressures on health care budgets, enhanced efficacy and safety of new pharmaceuticals, and the growing impact of generic products in the marketplace have held down rates of new drug approvals in spite of ever-increasing costs of pharmaceutical R&D. Discovery of new medicines has never been either easy or predictable, and especially so for the so-called "block buster" products that the industry has come to rely upon. Furthermore, the industry's prior 10-year focus upon its productivity, efficiency, cost, and cycle time inadvertently diverted attention away from its true "Achilles heel": quality, being that over the past two decades or more >90 % of promising candidate drugs entering regulatory toxicology (GLP) testing in preparation for clinical Phase I failed to subsequently gain marketing authorization (Cook et al. 2014). Going forward, safety pharmacology will be influenced in large part upon its ability to enhance the quality (and thus improve the probability of successful marketing authorizations) of new candidate drugs, alignment with training and interests of newly minted young scientists, and continuing evolution of scientific and technological advances and responding to regulatory challenges, including the license to practice necessary animal research in certain global territories.

As we have described elsewhere in this chapter, overall discovery and development of new pharmaceuticals has become increasingly challenging and that trend is

likely to continue into the future. While promising new therapeutic targets offer the hope of a favorable biology, risks exist that the new target will not produce an adequate therapeutic response in targeted patient populations or will be associated with unanticipated safety issues which are unabated. In addition, the costs of failure of a compound to reach the marketplace and the regulatory hurdles to registration are becoming more and more restrictive (Kaitin and DiMasi 2011; Munos 2009). The pharmaceutical industry's "inconvenient truth" is that quality has to be built in prior to selection of a candidate drug for first GLP dose, if not at a much earlier stage in the discovery phase of identifying that drug candidate. This is because once a candidate drug transitions into the development phase, further study of the candidate devolves to the equivalent of a high-stakes game of poker, wherein going forward only two decisions will ever be made—to invest further resources in the candidate (e.g., put more money in the "pot") or to quit development (e.g., "fold"). The point is that once a candidate drug is selected, its quality and probability of success is fixed. Indeed, further investment in safety pharmacology bioassays after first GLP dose will only contribute to causing a candidate drug to fail earlier, potentially saving further development costs, but also adding to the overall rate of compound attrition just the same. Thus, the greatest potential for positive impact of safety pharmacology in pharmaceutical R&D is in the discovery phase and involves safety pharmacology's ability to contribute to improved characterization and selection of higher-quality candidate drugs, thereby improving probability of eventual marketing authorization and decreasing overall pipeline attrition (see below). Having said this, safety pharmacology data can be applied in the context of risk management and risk mitigation. In this context, the data can be used in several ways which include but are not limited to (1) support regulatory approval, (2) investigate discrepancies that may have emerged within and/or between nonclinical and clinical data, (3) understand the mechanism of an undesirable pharmacodynamic effect, (4) provide reassurance for progression into multiple dosing in humans and/or large-scale clinical trials, and (5) assess drug–drug interactions.

But consider what safety pharmacology in discovery looks like.

1. Safety pharmacology bioassays. The ICH S7A Core Battery and secondary testing recommendations are animal bioassays for drug-associated adverse effects on organ functions. In discovery, particularly at lead identification and lead optimization stages, there is seldom sufficient quantity of compound, let alone time to conduct and report these sophisticated bioassays. In discovery therefore, safety pharmacology bioassays have to be compatible with the "design-make-test-analyze" cycle of the drug discovery process, needing to use smaller compound amounts and being relatively higher throughput, with short cycle times. Zebrafish embryos, Caenorhabditis elegans (*C. elegans*), and in vitro 2-D and 3-D cellular (e.g., "microphysiological systems") constructs would seem more likely candidates, if assays can be developed and, more importantly, qualified as appropriately sensitive and specific to identifying risk (Redfern et al. 2008). The purpose of these new assays would be to predict

significant outcomes in the present ICH S7A recommended assays, because only by getting through the current "gauntlet" of GLP safety pharmacology and toxicology testing may allow studies in Phase I volunteers and patients to commence.

2. Safety pharmacology mechanisms: Safety pharmacology has to become acquainted with and focused upon the molecular mechanisms (promiscuous molecular targets and vulnerable cellular pathways) that are responsible for drug-induced (AEs) on critical organ functions (see ICH S7A), just as efficacy pharmacology evolved from being bioassay-based to mechanism-based nearly a half century ago (Bowes et al. 2012). A "case study" of the impact of understanding mechanism in safety pharmacology is the example of blockade of the hERG channel and QT prolongation. Once it was understood that the vast majority of pharmaceuticals that were associated with drug-induced QT prolongation (e.g., drug-induced long-QT syndrome) did so by blocking the cardiac hERG channel, the science and conduct of these investigations changed rapidly: (1) focus upon the human delayed-rectifying cardiac potassium current (I_{Kr}) insured direct translation to human clinical outcomes; (2) high-throughput technologies to screen for drug–hERG channel interactions were developed; (3) conduct of these evaluations shifted from veterinary electrocardiologists to electrophysiologists and away from whole animals and cardiac explants (e.g., Purkinje fibers) to cellular transgene biology; (4) chemical–hERG channel structure activity relationships (SAR) guided chemical synthesis decisions to avoid predictable hERG channel interactions; and (5) in silico modeling of chemical–hERG channel interactions allowed drug designers to test compounds for hERG potential before committing to synthesis (Gavaghan et al. 2007). Indeed, there is reason to absolutely believe that the same pattern of activities will occur as we identify the molecular mechanisms underpinning drug-induced changes in cardiac contractily, seizures, bronchoconstriction, and kidney tubule nephrosis. This of course assumes a simple model of drug toxicity, as exemplified by blockade of the hERG channel. If successful, all that will be needed will be to identify those mechanisms, and the chemistries that underpin these adverse reactions, so that they can now be "designed away" in discovery. On the other hand, more complex mechanisms of toxicity will require more complex and sophisticated models that are not as easily adapted as the preclinical assays assessing a simple mechanism.

   That is not to say that the challenge of developing complex models of cardiovascular function are unachievable and cannot be overcome. Recently, researchers at Harvard's Wyss Institute have reported developing microchips comprising the culture cell systems that demonstrate three-dimensional functionality of human organs (http://wyss.harvard.edu/). These "organs-on-a-chip" represent an advance that could prove revolutionary for pharmaceutical companies both from an efficacy and safety point of view. Dynamic and temporal measurements of cardiac, vascular, and pulmonary function are achievable today using these emerging technologies. Similarly, the utilization of the semiautomated in vitro hippocampal brain slice assays for assessing the seizure

liability of compounds is emerging with some promising data (Easter et al. 2007).

3. Safety pharmacology in silico modeling: In silico approaches, using computational models, have been developed to predict the potency of new chemical entities at cardiac ion channels (Gavaghan et al. 2007). Mathematical models of the human ventricular action potential are being developed based on activities at cardiac ion channels. Computer modeling of the heart from genes to cells to the whole organ is becoming a reality (Davies et al. 2012; Noble 2002, 2004; ten Tusscher et al. 2004). In vitro approaches using adult human stem cell-derived cardiomyocytes are also being advanced. These cells have some of the characteristics of differentiated ventricular cardiomyocyte including a typical action potential. The ability to culture and expand human adult stem cells in vitro provides potential for producing quantities of cardiomyocyte progenitors which could be employed to assess the effects of new chemical entities on cardiac ion channels, electrical activity and contractility, and structural cardiotoxicity (Lamore et al. 2013; Pointon et al. 2013; Qu et al. 2013). As per the hERG "case study" cited above, once the specific proteins responsible for drug-induced AEs on organ functions are known, the human homologues and translation of AEs identified, then structure activity relationships can be developed and modeled, permitting in silico evaluation of adverse effect potential before new compounds are synthesized. As described earlier in the chapter, an integrated approach has been proposed by a consortium of FDA, HESI, and CSRC to rely on an algorithm incorporating data from in silico modeling of results from the study of new chemical entities on an assortment of critical cardiac ion channels and the addition of data collected in iPSC cardiomyocytes to predict proarrhythmic risk posed by the new chemical entities (Cavero and Holzgrefe 2014; Chi 2013; Sager et al. 2014).
4. Safety pharmacology discovery scientists: As is apparent from the discussions of the scientific and technological advancements that are quickly becoming the important tools of safety pharmacology, the types of scientific training and background necessary to work in the field in the discovery phase are very different than that needed to conduct in vivo GLP bioassays in the development phase. Cellular and molecular pharmacology, bioengineering, and informatics skills will be at a premium and much more closely matched to the training of young scientists graduating from academic programs today. Our challenge in working with these young investigators is to leverage the experience of bridging the results of sophisticated assays to developing a risk assessment and formulating a judgment of whether to advance a new target or novel test agent into the clinic.

Also consider those imposing questions/changes that are more broadly shaping safety pharmacology.

1. Regulations governing safety pharmacology: As noted elsewhere in this chapter, the ICH S7A and S7B guidances have been in place for nearly a decade or more,

and currently there is no activity for revision or withdrawal of these documents. Therefore, the testing schemes set forth in these documents will likely remain in place for new pharmaceutical agents positioning for Phase 1 clinical trials for the foreseeable future. However, as summarized in Fig. 1, there has been a growing number of guidelines issued referring either entirely or in part to safety pharmacology, reflecting an increase in stakeholders concerns. For example, a guidance focusing on the "nonclinical investigation of the dependence potential of medicinal products" has been released that will influence the overall approach to safety pharmacology especially for CNS-targeted new molecular agents, but also for those compounds which are being developed for other indications, but with an ability to penetrate the CNS (Anon 2010a). Moreover, safety pharmacology is considered an important component to emerging regulatory guidance such as the "safety evaluation of pediatric drug products and nonclinical studies for development of pharmaceutical excipients." The discipline is considered integral to the evolving regulatory strategies for safety to contribute to the acceleration of the introduction of new molecular entities into clinical phases. In its Critical Path Report (Anon 2004a), the FDA suggested that limited exploratory Investigational New Drug (eIND) studies in humans can be initiated with less or a different type of nonclinical support, including a modified safety pharmacology component, because eIND studies present fewer potential risks than do traditional Phase I studies that look for safety, tolerability, and dose-limiting toxicities (Sibille et al. 2010). The ICH M3 (R2) guideline, published in 2009, further expanded the exploratory clinical trial approach with international consensus. In these cases, the safety pharmacology program should be considered on a case-by-case basis dependent on the specific objectives for a given exploratory clinical trial and any known concerns regarding the molecule and its therapeutic target (Anon 1997b, c).

2. Translation to the clinic: Beyond the promises of novel targets and emerging tools being revealed by molecular biology, the translation of findings in preclinical models to the realization of significant AEs in humans, recognized or unrecognized prior to clinical trials, has been and will continue to be a significant challenge to our discipline and more generally to fields of safety sciences (Bass et al. 2009). The examples of molecules whose use was curtailed or completely discontinued because of safety concerns simply point to our inability to model all human AEs in a preclinical setting (Bass et al. 2009). In terms of translation of pharmacodynamic activity of the cardiovascular, central and peripheral, respiratory, renal, endocrine, and other biologic systems (Olson et al. 2000), our ability to qualitatively predict human toxicity or to monitor for potential adverse events is achievable in many, but not all, cases when the data are laid out organ system by organ system, as cited in the prospective surveys of Owen (1962), Schein et al. (1970), and Valentin et al. (2009). The guidelines on drug dependence and abuse liability previously mentioned are based on models that demonstrate a translational link to clinic (Anon 2010a). The use of novel genomic and proteonomic biomarkers has proven useful, or has potential to be used, in monitoring for organ system toxicity (e.g., cardiovascular toxicity

biomarkers, renal toxicity biomarkers, etc. (Benjamin et al. 2015; Cove-Smith et al. 2014; Osaki et al. 2014; Ozer et al. 2010). In this regard, many of the measures of pharmacodynamic toxicity employed in safety pharmacology are either a routine measure in clinical studies or can be adapted to the design of clinical studies in special situations. However, recognizing the importance played by biomarkers, more sophisticated approaches to identifying models of pharmacodynamic toxicity that translates to humans remain an important objective of safety pharmacology.

In this regard, safety pharmacologists are being asked by the clinical community and the pharmaceutical industry to identify small, clinically relevant changes in biology (e.g., 2–3 mmHg change in blood pressure) and to predict clinical outcomes that are only seen following chronic exposure or a lifetime of treatment with a test agent. However, safety pharmacology's long-term vision should not be to perpetuate, but eventually to "retire" those bioassays that can be replaced by more specific and translatable mechanism-based assays. Thus, safety pharmacologists (or professional organizations such as the different societies of safety pharmacology) should engage in periodic dialog with their stakeholders (e.g., regulators, clinicians, and patients) who are currently using the data from the in vitro and in vivo bioassays, to ask for what purpose and what decisions are data being used in order to judge their value to inform and contribute to their deliberations (Robinson et al. 2008). On a related note, efforts to construct databases relating predictions based on nonclinical assays to the clinical experiences in humans should continue (Trepakova et al. 2009; Valentin et al. 2009).

3. Coalescing of Preclinical GLP Testing Centers and Scientists: In the meantime, the current ICH-recommended safety pharmacology bioassay expertise appears to be coalescing in large GLP preclinical testing centers, increasingly within CROs. This is entirely rational as these are sophisticated bioassays that require a high degree of training, skill, and technological expertise to execute and, as mentioned above, is consistent with a diminishing pool of trained young scientists in some territories (notably the EU and USA). In the coming years, we anticipate further consolidation of the ICH-directed GLP safety pharmacology bioassays in those territories supportive of in vivo animal research. We also anticipate that these centers will be focused on either producing trained investigators or importing them from other parts of the world.
4. New Paradigms to Treat Diseases: To date, safety pharmacological evaluations have been mostly focused upon ensuring the safety of human volunteers participating in Phase 1 clinical trials of small molecules. That mission has expanded somewhat in the past decade to include biologics including hormones and cytokines, antibodies and oligonucleotide constructs (Anon 1997b, c), and human patients participating in Phase I clinical trials in oncology (Anon 2010b). Going forward, safety pharmacology will face further challenges, many that have been described in this chapter, but also attempting to predict AEs on critical organ functions associated with cell-based therapeutics, fixed combination

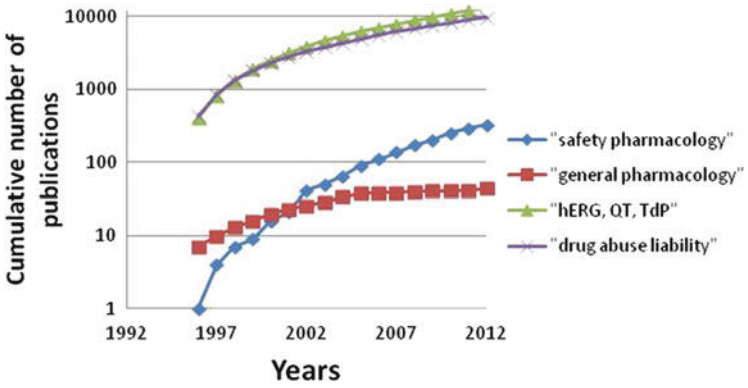

Fig. 4 Trends in the number of publications referring to "general pharmacology" (*square symbols*), "safety pharmacology" (*lozenge symbols*), "hERG, QT, TdP" (*triangle symbols*), and "drug abuse liability" (*cross symbols*). Over the last 15 years, in contrast to general pharmacology, there has been a steady increase in the number of publications relating to safety pharmacology. Moreover, there have been a large number of publications increasing steadily on "hERG, QT, TdP" and "drug abuse liability," two areas of interest to the safety pharmacologist

products, and those events that only appear in the presence of specific underlying disease states.

The discipline of safety pharmacology has provided a platform in which to facilitate the development and dissemination of enabling technologies to address the concerns associated with novel molecular targets. Advances in molecular biology and biotechnology allow for the identification of new molecular targets, leading to the discovery and development of newer pharmaceutical agents that act at these newly discovered sites in an attempt to ameliorate the disease. Moreover, therapeutic approaches are being developed (e.g., gene therapy, microRNAs) that offer the promise of treating disease but also pose new challenges to safety pharmacology in adequately mitigating the risk of potential pharmacodynamic toxicity. Inherent in the novelty of new targets is the risk of unwanted side effects that may or may not be anticipated based on current scientific knowledge and with current technologies and model assays. As illustrated by the growing number of publications on safety pharmacology-related topics (Fig. 4), the discipline faces significant challenges to keep pace, adapt, and incorporate the latest scientific knowledge and novel technologies in the study of new molecular agents to identify those effects that pose a significant risk to human volunteers and patients. This is a challenge to be proactive rather than reactive to the rapidly changing environment that surrounds each and every one practicing in the field of safety pharmacology.

Many pathological conditions are multifactorial and involve multiple and, in some cases, redundant pathways and comorbidities. Therefore, in some cases such as functional gastrointestinal disorders, infectious diseases, or in oncology, there is recognition that combinations of drugs provide significant advantages for new treatments. As a consequence, "nonselective" drugs acting simultaneously at

multiple targets might be considered for clinical development, which will require a more careful assessment of their potential to induce additive or synergistic ADRs.

On a related note, demand for fixed-dose combination products that target multiple mechanisms by individual entities leading to amelioration of disease is expected to increase, representing a significant proportion of the overall sales of medicinal products. Fixed-dose combinations primarily focus on the combinations that include one or more established products combined with one or more new molecular entity(ies). The testing strategies and options are outlined, e.g., in the ICH multidisciplinary guideline M3 (R). Some of the key questions that will need to be addressed regarding fixed-dose combinations include, but are not limited to: (1) how many safety pharmacology packages will be needed, (2) how will a risk assessment be conducted, and (3) how will the safety margins principle be applied? Also important to consider is the timing of such studies in relation to clinical development in order to contribute to the potential for success of a new therapy. These and other questions related to the study of nonselective test agents or fixed-dose combinations will be answered by scientists as they engage in the development of scientifically based strategies directed at understanding the properties of the individual agents, as well as compounds given in combination.

6 In Summary

The next decade offers safety pharmacology many challenges but also hope and opportunities for continued growth and great aspirations. In this effort, the Safety Pharmacology Societies (Safety Pharmacology Society, Japanese Safety Pharmacology Society, and the emerging Chinese Safety Pharmacology Society) will play increasing roles in supporting the short- and long-term future developments of the discipline. In the future these organizations should continue to serve the local societies and the greater good of mankind by contributing to the improvement of the way in which new safe and effective medicines are discovered and developed.

The progress of addressing critical issues is slow, and one hindrance is the availability of technological resources and data upon which to elaborate on a scientific principles or questions that are important to safety pharmacologists. To facilitate the sharing of these experiences and learnings, consortia and institutional collaborations have emerged under a number of umbrella organizations offering a model upon which to emulate similar efforts as those which are carried out within the Safety Pharmacology Society. This has occurred on a limited scale already in prior initiatives sanctioned by the Society (Bass et al. 2011; Guth et al. 2009; Leishman et al. 2012; Vargas et al. 2008), but the opportunity exists to see this model expanded to other significant issues challenging the field. Indeed, as noted earlier in this chapter, this continues to occur in a number of different forum (e.g., HESI, BfArM European Concordance Project, ABPI-Animal Model Framework, TI-Pharma PK/PD Assessment, and CSRC). As well, there may be other opportunities for collaborations among different umbrella organizations who are funded by the pharmaceutical industry; this is particularly important at this time as the availability of funding sources has become more and more limited. CiPA is a

good example of bringing together societies, institutions, and consortium in order to address a crucial issue facing the pharmaceutical and regulatory communities. The other advantage of these collaborations is the potential to identify ways of sharing safety data across the industry and leveraging experiences among the scientists as a way of accelerating progress of addressing significant issues impacting human health (Munos and Chin 2009). The societies by providing a long-range vision of the future of safety pharmacology should be the natural platform for cooperation and collaboration between scientists.

Ultimately, increases in scientific knowledge and development of new technologies may lead to the development of new, robust, reliable and reproducible assays and models for predicting ADRS that are currently poorly predicted, such as side effects difficult to identify under preclinical conditions, e.g., headache or suicidal ideation. In this regard, it would be wise to concentrate efforts on ADRs having either high incidence or high impact on human safety. To that end, safety pharmacologist cannot ignore any scientific and technological innovation that has the potential of improving our ability to detect, to predict, and wherever feasible to eradicate human safety threats.

While a worldview of safety pharmacology past, present, and future will be informative, it is important to be cognizant that this account of history is a "cumulative snapshot in time." Over the past 15–20 years, several authors have published similar reviews of the field of safety pharmacology, also as brief snapshots in time (Bass et al. 2004b, 2011; Kinter and Valentin 2002; Lindgren et al. 2008; Redfern and Valentin 2011). As you read about the history and present and future challenges that face us at an ever-changing and dynamic time, let us appreciate that the future will be laid by the current practitioners and the next generation of safety pharmacologists who will be asked to provide insight and direction to those events which will define the history of this rapidly evolving field.

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In Vitro Early Safety Pharmacology Screening: Perspectives Related to Cardiovascular Safety

Gary Gintant

Contents

Abstract

In vitro screening for cardiovascular safety liabilities of novel drug candidates presents a challenge for the pharmaceutical industry. Such approaches rely on detecting pharmacologic effects on key components of complex integrated system early in drug discovery to define potential safety liabilities. Key to such studies are the concepts of hazard identification vs. risk assessment, drug specificity vs. selectivity, and an appreciation of the challenges faced when attempting to translate in vitro findings to preclinical in vivo as well as clinical effects. This chapter defines some key aspects of early safety pharmacology screening for cardiovascular liabilities, citing studies of two key depolarizing cardiac currents (fast sodium current and L-type calcium current) as examples linked to effects on cardiac conduction and repolarization.

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Cardiac conduction and repolarization • Hazard identification • Risk assessment • Selectivity • Sodium current • Specificity

1 Introduction

An urgent need remains to reduce attrition in later stages of drug discovery. Aside from business considerations, attrition arises from two sources, namely, efficacy and safety concerns. For a drug to be successful, it must demonstrate efficacy along with acceptable safety. In the broadest contest, safety refers to overall drug effects, which include (a) potential on-target adverse effects (side effects consistent with a drug's known mechanism of action), (b) off-target effects (side effects not related to a drug's known or targeted mechanism of action), or (c) drug–drug interactions (metabolic interactions increasing the levels of the drug or concomitant medications). The balance between efficacy and safety forms the basis of the therapeutic index, defined as the ratio of the highest exposure to the drug that results in no toxicity to the exposure that produces the desired effectiveness (see a recent review by Muller and Milton 2012). Safety considerations can span across toxicologic studies (based more on form and structure) as well as functional studies (including more acute safety pharmacology studies).

The field of safety pharmacology employs the basic principles of pharmacology to provide data useful in evaluating risk/benefit assessments of evolving drug candidates (see Pugsley et al. 2008). In general, safety pharmacology studies evaluate functional endpoints, as compared to "classical" toxicology studies that focus more on morphological endpoints. More traditional safety pharmacology studies (see Bass et al. 2015) focus on acute in vivo studies ensuring safety of vital organ systems ("to identify undesirable pharmacodynamic properties of a substance that may have relevance to its human safety") as outlined in the ICH S7A regulatory guidance (US FDA ICH S7A 2001). These studies typically evaluate later-stage discovery compounds (destined for first in human clinical studies) and performed under good laboratory practice (GLP) conditions to fulfill regulatory requirements. A newer area of safety pharmacology has since evolved whose goal is to identify potential hazards and risks of evolving drug candidates. The terms "Exploratory Safety Pharmacology" (Bass et al. 2009; Cavero 2009a, b) and "Frontloading" (Pugsley et al. 2008) have been used to describe these safety studies performed prior to or during lead selection. These studies, often performed in vitro, allow for reduced compound requirements (typically a few milligrams vs. hundreds of milligrams for in vivo studies) as well as reduced cost, animal usage, and more rapid turnaround times. Frontloading of early safety studies saves time and resources by removing compounds with potential liabilities early, informs project teams of potential toxicities, and guides further preclinical exploratory studies and clinical risk mitigation strategies with the goal of reducing late-stage attrition.

This chapter will present some perspectives on in vitro exploratory safety pharmacology studies, briefly discussing some strengths and important limitations of early safety screening. Some off-target cardiovascular safety assays will be cited, including binding assay studies, computational methods, and functional ion channel screening (emphasizing two prominent depolarizing cardiac currents, namely, fast sodium (I_{Na}) and calcium ($I_{Ca,L}$); key potassium currents are covered elsewhere in this volume). Emphasis within this chapter will focus on effects of small molecules, rather than biologics or biopharmaceuticals. For this later emerging class of therapeutics, adverse effects are more likely to be on-target-related and immune system-induced or arise with longer-term exposures (Kooijman et al. 2012; see Vargas et al. 2015) consistent with their greater specificity, more limited distribution, and longer half-lives (such as monoclonal antibodies) compared to small molecule therapeutics.

2 Consideration of Hazard Identification vs. Risk Assessment in Exploratory Safety Studies

It is informative to consider the early safety studies in drug discovery using the concepts of hazard identification and risk assessment. Borrowing definitions from the field of environmental risk, a hazard may be defined as a chemical's *intrinsic ability to cause harm* or produce adverse effects. In contrast, risk is associated with the *probability of harm or adverse effects* will occur at select concentrations and scenarios. Bottled propane (as an example) represents a recognized hazard that has a low probability of causing harm when used properly; thus, the risk assessment depends on the amount of propane stored (concentration), conditions for storage, and use. Similarly, a novel drug candidate can be considered in regard to potential hazard and risk assessment.

In exploratory safety studies, hazard identification is often conducted during early selection of lead candidates. These studies serve to inform of potential risks and are used internally for decision-making, rank ordering of compounds, and subsequent lead optimization efforts. In general, early in vitro assays are conducted with simple preparations (transfected cells or membranes) that represent the most simplest of drug–receptor interactions (reflecting hazard identification). As these are reductionist approaches, they need not necessarily reflect functional effects on either cells, tissues, organs, or whole animals in normal or diseased states [representing more complete (and complex) risk assessment]. In contrast, later evaluations of risk assessment generally necessitate more complex assays (ranging from cell-based in vitro studies to ex vivo and organ studies). Such studies will subsequently include in vivo whole animal studies that form traditional safety pharmacology packages satisfying regulatory authorities. For example, an evolving compound may be shown to bind to beta-adrenergic receptors by ligand-displacement studies, thus identifying a potential hazard. Subsequent (follow-up) risk assessment requires further studies that could include functional in vitro studies involving evaluation of effects on heart rate and cardiac contractility (e.g., in

isolated atrial preparations). Results from these efforts might indicate beta-receptor agonist effects (increased heart rate and cardiac contractility (depending on receptor–effector coupling and binding affinity)) or antagonist effects (decreased heart rate and contractility in the presence of beta-adrenergic stimulation). One critically important contribution of the safety pharmacologist is to recognize the strengths and limitations of early exploratory studies and their translation to more integrated preclinical (e.g., whole animal) and clinical studies.

The value of information gained from early exploratory safety pharmacology studies depends on the stage of drug discovery and available resources. If multiple drug candidates are under evaluation during early lead optimization, it might be prudent to eliminate the most conspicuous (potent) "offenders" based on rank ordering of results centered on hazard identification, thus saving resources for subsequent testing of remaining drug candidates. In contrast, if only a few drug candidates are available (e.g., after efficacy targets are achieved), then downstream studies with more complex, integrated systems focused on risk assessment would be warranted. The value of early hazard identification studies also depends on the extent (and timing) of subsequent risk assessment studies. A compound identified with a potential hazard (but with promising preclinical efficacy) might be pursued further should later risk assessments demonstrated limited liabilities at exposures where efficacy is anticipated. Clearly, care must be taken to avoid overinterpreting the extent of risk in order to avoid discarding promising compounds early in the drug pipeline that are in fact safe and would conceivably be eliminated during later safety testing. Finally, the combined results from hazard identification and subsequent risk assessment studies would both provide confidence in designing later risk mitigation strategies for compounds deemed worthy of further progression.

3 Specificity and Selectivity

One of the key strengths of early safety studies is the ability to identify potential off-target pharmacologic activity during the lead selection process. This process should occur concurrently with activities focused on identifying the best lead candidates. Broad profiling of activities, if conducted early, provides for the efficient attrition of multiple compounds and allows for more efficient medicinal chemistry efforts focused on lead optimization.

Two key concepts to consider regarding early compound profiling are specificity and selectivity. While different definitions may be found, I will refer to specificity (derived from Latin "species") as describing a drug's ability to exert a single effect by a single mechanism of action. In most cases, drugs are described based on their selectivity across multiple receptors, as most drugs are not specific and will interact with more than one receptor (especially at higher exposures). Single and multiple effects that may result from drug interactions with one receptor are referred to as "on-target" pharmacology. Indeed, on-target pharmacology may result in intended and adverse effects and often leads to understanding the physiology of affected

systems. The antihistamine diphenhydramine provides such an example. As an antagonist at peripheral H1 receptors, this drug mitigates the effects of histamine release, thus providing relief from common allergies. However, as an antagonist at H1 receptors in the central nervous system, diphenhydramine produces drowsiness (an on-target effect sometimes used to benefit).

Effects shared by drugs with the same mechanism of action are referred to as "class effects" or "class actions." Early safety pharmacology studies are useful in defining class effects related to potential safety issues and may prove critical in discerning the overall value of novel drug targets. A drug's overall selectivity (that defines its "off-target" pharmacology) can be described based on differences in the dose–response curves for the multiple receptors or responses involved. It is preferable for a drug to exert its therapeutic actions (on-target effects) at lower exposures than for adverse or untoward effects elicited at off-target receptors, especially if the off-target effect is linked to a serious adverse effect (thus forming the basis for a therapeutic margin). However, when multiple receptors are involved in defining an overall effect, it is possible that both may define the overall safety profile. For example, it is postulated that the liabilities linked to hERG current block (proarrhythmia resulting from delayed ventricular repolarization due to reduced outward current) may be offset by concomitant block of L-type calcium current (which may act to shorten ventricular repolarization as a result of reduced inward current). These two opposing forces may balance, thus mitigating the potentially dangerous QT prolongation observed with hERG blockade alone (see Fermini and Fossa 2003 for discussion).

The weight-loss drug lorcaserin provides a recent example of a drug overcoming a selectivity-based safety liability. Lorcaserin was approved in 2012 as an adjunct to diet and exercise for chronic weight management in adult patients as well as patients overweight who also have at least one weight-related comorbidity. Lorcaserin is described as a selective 5HT2C serotonin receptor agonist that leads to GPCR-linked cellular excitatory activation that elicits accumulation of inositol phosphates and downstream activation of phospholipase C. Activation of the 5HT2C receptor has been implicated in feeding, and knockout mice have demonstrated hyperphagia and an obese phenotype. However, the 5HT2C receptor is one of at least 13 distinct 5-HT receptor subtypes cloned and characterized (see Hoyer et al. 2002). Heart valvulopathy, a significant adverse effect of an earlier weight-loss drug combination fenfluramine/dexfenfluramine, has been linked to off-target activation of 5HT-2B receptors in clinical and preclinical in vivo studies (Rothman et al. 2000; Elangbam et al. 2008; see Hutcheson et al. 2011 for a recent review). Selectivity of lorcaserin for the 5HT2C receptor was demonstrated to be 8–15× vs. the 5-HT2A receptor and 45–90× vs. the 5HT-2B receptor lending support for subsequent drug development and eventual approval of lorcaserin (FDA Lorcaserin Briefing Document 2010).

Broad profiling to characterize selectivity provides useful data required to prioritize candidates for further testing as well as guide follow-on studies to inform risk assessment. The extent of early profiling of compounds often depends on the resources available. Multiple contract research organizations offer in vitro

screening services based on binding assays for receptors (GCPRs, nuclear receptors, kinases, etc.), enzymes, ion channels, and transporters; examples of such protocols can be found in such journals as *Current Protocols in Pharmacology*. Most early protocols rely on a competition assay model using radiolabeled ligands and either of three assay formats (filtration, scintillation proximity assay, and centrifugation); assays are typically optimized to provide fast, consistent, and reproducible results. One potential strategy is to initially screen compounds at a single high concentration (e.g., 1 or 10 μM), with follow-up studies on hits for IC50/Ki determinations when compounds display more than 50 % inhibition of control value or 50 % stimulation relative to control. Subsequent studies linking binding results to functional responses are required to effectively translate receptor studies and inform potential off-target effects, as both binding affinity and efficacy drive functional effects in the simplest of cellular systems (the next level of integration). Binding studies provide little information regarding specifics of drug–target interactions (e.g., agonism, competitive or noncompetitive antagonism, allosteric modulation, reverse agonists, desensitization, etc.) that may require further clarification.

While established in vitro studies have traditionally focused on simpler subcellular assays, a growing number of whole cell-based screens are emerging. Such assays are likely useful for evaluating the integrated cellular response to a drug, representing multiple (and likely interdependent) effects on multiple cellular proteins and machinery, metabolic status, and other factors. It is the combination of these multiple factors that determines a drug's overall efficacy or safety profile. Indeed, a drug may have different effects/efficacies [termed pluridimensional efficacy (Galandrin and Bouvier 2006)] dependent on the type of assay/assay system and conditions used [see Kenakin and Christopoulos (2012) for a recent review related to G protein-coupled receptors]. A potential complication of translation of in vitro cellular responses is provided by studies of seven transmembrane receptors that can form many conformations of the receptor, leading to behaviors where ligands can stabilize unique conformations that provide for selective activation of signaling pathways (termed "biased ligands"; see Kenakin 2011 for a review). G protein signaling vs. beta-arrestin recruitment to the parathyroid hormone receptor provides an example of a biased ligand (Gesty-Palmer et al. 2011). Thus, a specific assay to detect a ligand may report only a subset of efficacious compounds based on the assay conditions and measured endpoints. As compared to more simplified test systems, whole cell assays (e.g., using native cells or human-derived stem cells) may be less prone to demonstrate confounding bias as the integrated cellular response reflects the integrated response of multiple signaling pathways. The complexity inherent in cell-based screening approaches might be expected to differentiate subtle ligand effects (at the expense of making interpretation of cellular mechanisms and affected pathways more difficult).

A recent study by Loukine and colleagues (2012) described a computational approach to predict unintended "off-target"-based adverse drug reactions. After characterizing structural similarities of known ligand molecules for 73 biological targets, the authors searched for structural relationships of groups of ligands

compared to test compounds. (This approach is somewhat different from other conventional methods that determine the strength of drug–receptor interactions based only on ligand and receptor structures.) Approximately half of the off-target predictions were true, with affinities of the new off-targets ranging from 1 nM to 30 μM. Of the 656 marketed drugs tested on the 73 "side-effect" targets, each drug modulated an average of seven safety targets, with more than 10 % acting on approximately half of the targets. Finally, based on the drug set tested, the 10 most promiscuous targets identified were (in descending order) Nav1.5 cardiac sodium channel, 5-HT_{2B} serotonin receptor, 5-HT_{2A} serotonin receptor, α_{2a} adrenergic receptor, 5-HT_{1A} serotonin receptor, α_{1A} adrenergic receptor, M_2 muscarinic receptor, hERG (I_{Kr}) potassium channel, H_2 histamine receptor, and D_4 dopamine receptor. With the exception of Nav1.5 and hERG (both ion channels), most promiscuous targets were G protein-coupled receptors, followed by transporters; enzymes, nuclear receptors, and ligand-gated ion channels were less promiscuous, and peptide-recognizing receptors were identified the least. This method provides an example of an early computational prediction of off-target interactions that can guide subsequent selection of appropriate interrogative in vitro and in vivo screening studies (should the compound be synthesized). However, given the high rate of false-positive findings with this approach, it should be considered as one component of a more complete early safety screening exercise.

Finally, it is informative to (re)evaluate exploratory safety pharmacology studies when a potential safety signal emerges from later preclinical studies (e.g., GLP-based assays to satisfy regulatory authorities) or subsequent clinical studies. Such reviews are informative regarding defining assay performance, which can include either the presence or absence of responses (concordance vs. discordance) as well as sensitivity of responses in preclinical vs. clinical studies (e.g., graphically using concentration–response relationship). Conversely, a preclinical signal not validated in clinical studies should be interrogated in order to establish potential mechanisms (e.g., species differences), as well as to inform on the level of translatable risk involved. Such studies are essential in evaluating the safety of subsequent backup candidates.

4 Exploratory Safety Pharmacology Studies with Cardiac Channels Involved in Cardiac Conduction

Screening of cardiac ion channels (beyond the expected regulatory hERG current screening) is assuming a more prominent role in early safety pharmacology studies. This greater emphasis arises from multiple sources, including familiarity with the prominent role of hERG screening (as detailed in the US FDA ICH S7B Guidance 2005), growing knowledge and characterization of inherited ion channelopathies linked to proarrhythmia, greater attention to observed ECG changes now evaluated as part of evolving thorough QT studies, counterscreening of cardiac channels based on homologies of noncardiac channel targets from various therapeutic areas, and a growing awareness of cardiotoxicity with oncology therapeutics.

Enabling technologies for expressing ion channels in various systems and the growing number of automated patch clamp platforms for functional studies (see Möller and Witchel 2011) provide further impetus for such studies. Often subtle differences between different channel isoforms (of targets vs. non-targets, e.g., among the nine recognized sodium channel isoforms) necessitate counterscreening early to provide efficient compound differentiation in lead optimization. The following sections will discuss the basis for screening of two inwardly directed (depolarizing) cardiac ion currents that play a prominent role in defining conduction and the action potential plateau of ventricular myocardium, namely, sodium (INa or Nav1.5, encoded by the SCN5a gene), and calcium (ICa,L, or Cav1.2, encoded by the CaCNA1C gene). Despite the importance of these channels in maintaining normal cardiac rhythm, their evaluation is not specifically covered in current regulatory guidances. The reader is referred to Chap. 7 in this book for discussion of hERG screening.

5 Fast Sodium Current

In working atrial and ventricular tissues, fast inward sodium current flowing through Nav1.5 channels is responsible for the action potential upstroke. These voltage-dependent channels rapidly activate (open) and inactivate (close) over the time course of a few msec, producing a strong and transient depolarizing inward current. The rapid current kinetics, along with large current density, provides for rapid, non-decremental conduction through working myocardium. As would be expected, block of this current results in slowed conduction, manifest as prolongation of the QRS duration (a measure of ventricular conduction) and prolongation of the PR interval (reflecting the combined effects on atrial conduction and conduction through specialized ventricular conduction pathways (Purkinje fibers) leading to working myocardium).

Reduction of fast inward sodium current is generally considered a risk factor for proarrhythmia. The Cardiac Arrhythmia Suppression Trial (CAST) demonstrated increased mortality in post-myocardial infarction patients when treated with either of the three local anesthetic-type antiarrhythmic agents (flecainide, encainide, and moricizine) to suppress ventricular premature depolarizations (Epstein et al. 1993; CAST investigators 1989; Echt et al. 1991). Sodium current block may also lead to negative inotropic effects due to reduced intracellular calcium concentrations affected by the cardiac Na^{+}/Ca^{++} exchange pump (Ito et al. 1996), though this relationship is complex. Prolongation of the QRS duration in the presence of cardiovascular disease likely reflects pathophysiologic progression that is linked to proarrhythmia (see Nada et al. 2013 for a review). However, the relationship between QRS prolongation and proarrhythmia in patients without cardiac pathology is uncertain.

Block of cardiac sodium current by small molecules is complex, as drug binding (and hence channel block) is modulated by the state of the channel (that affects drug affinity as well as drug access). Consideration of the dynamic interactions between

drug (which may exist in charged and uncharged forms and may gain access to binding site(s) via different pathways) and various channel configurations (as the channel cycles through resting, open, and inactivated states with each heartbeat) is provided by the modulated receptor or guarded receptor hypothesis (Hondeghem and Katzung 1977, 1984; Hille 1977; Starmer and Courtney 1986). The extent of channel block is typically dependent on the rate of stimulation or electrical activity (use-dependent block) and the voltage prior to initiation of the upstroke (voltage-dependent block). Underlying these effects are time- and voltage-dependent modulations of the rates of drug association and dissociation. Thus, the extent of block is dependent on the rate and pattern of electrical activity, as well as the kinetics of drug-channel interactions These concepts are not new; using microelectrode techniques and measures of maximum upstroke velocity (Vmax), use-dependent block was first demonstrated with guinea pig papillary muscles treated with quinidine (Johnson and McKinnon 1957), and voltage-dependent block was described by Chen et al. (1975). While not necessarily sophisticated, techniques used in these early demonstrations still prove useful for characterizing local anesthetic effects of drugs in native cardiac preparations, with results more easily translatable to the intact heart. One of first demonstrations of voltage-dependent block using voltage-clamp techniques was shown by Bean et al. (1983) who demonstrated slowed sodium channel reactivation (recovery from block of cardiac sodium current) with lidocaine. In this study, potency of block at depolarized potentials (−65 mV approx. 10 μM) was significantly less than observed at hyperpolarized potentials (−120 mV, approx. 400 μM). Considering the multiple factors shown to modulate block of cardiac sodium current, one should recognize the limitation of a single IC50 value associated with one specific protocol to characterize block of cardiac sodium current.

In general, the kinetics of recovery from block determines the extent of block and defines three different subgroups of Class 1 cardiac drugs demonstrating local anesthetic effects within the Vaughan Williams classification (Vaughan Williams 1975, 1984; Nattel 1991). Thus, Class IA antiarrhythmic drugs (such as quinidine) slow the rate of rise of the action potential (and slow conduction) as a result of intermediate kinetics of association and dissociation from different states of the sodium channel. In contrast, Class 1B drugs (such as lidocaine) have little effect on the rate of depolarization or QRS duration due to rapid dissociation from sodium channels. Finally, Class IC agents (such as flecainide) markedly depress the rate of rise of the action potential and cause marked conduction slowing due to slow dissociation kinetics of drug from the channel (longer-lasting, cumulative block).

With the advent of automated patch clamp systems and heterologous expression systems, characterizing drug block of sodium current is easily accomplished (e.g., see Harmer et al. 2008; Penniman et al. 2010; Kirsch 2010). A more integrated assessment of local anesthetic effects that employs multiple approaches may provide more confidence regarding translation of early preclinical findings. A recent study demonstrated the ability to differentiate between “good” (lidocaine-like) and “bad” [flecainide-like (strong use-dependent block)] sodium channel-blocking activities using multiple in vitro approaches activities (block of human

cardiac INa in transfected CHO cells), rabbit Purkinje fibers (measuring upstroke characteristics), arterially perfused left ventricular wedge preparations (measuring QRS/conduction velocity), and rabbit Langendorff hearts (Lu et al. 2010). Another recent study illustrated an integrated approach for the preclinical evaluation of evolving drug candidates on cardiac conduction (with experimental elements including patch clamp studies, QRS interval measures in isolated Langendorff preparations, and PR and QRS measures in dog or nonhuman primate; see Erdemli et al. 2012). The in vitro studies by Lu et al. also demonstrated the enhanced effect of flecainide with myocardial ischemia, confirming prior findings that flecainide results in more marked depression of conduction in ischemia/reperfused myocardium (Kou et al. 1987). This confirmation highlights the utility of benchmarking and mechanistic studies when assessing proarrhythmic risk of more potent sodium channel-blocking agents.

Translating the various preclinical findings characterizing sodium current block to clinical findings represents yet another challenge. A recent study by Harmer et al. (2011) explored the relationship between drug-induced block of cardiac sodium current and QRS duration effects described in clinical literature. Specifically, they compared safety margins for 98 compounds (defined as the ratio of IC50 values for hNAv1.5 block/free Cmax based on clinical exposures) vs. reported QRS prolongation. They reported that QRS prolongation occurred, on average, at free plasma levels 15-fold below the calculated safety margins. Similarly, (1) a recent study by Heath et al. (2011) demonstrated that free plasma concentrations of flecainide and mexiletine 6–30-fold below IC50 values for block of hNav1.5 were sufficient to prolong the QRS interval/duration by 10–20 % in preclinical and clinical studies, and (2) an abstract by Cordes et al. (2009) concluded that free plasma concentrations approx. 3–11-fold below the IC50 values for sodium current block were sufficient to produce QRS widening.

These above translational studies are somewhat surprising, considering the high current density of hNav1.5 in ventricular myocytes (and presumably high "depolarization reserve"; see Gintant et al. 2011). Indeed, computer simulations of cardiac propagation describe a nonlinear relationship between conduction and reduced maximal sodium current conductance, with a 50 % decrease in conductance resulting in only modest reduction of conduction velocity from 55 to 32 cm/s (Shaw and Rudy 1997). This study also suggested that extreme QRS prolongation is necessary for intraventricular conduction failure in normal hearts. The sensitivity of ventricular conduction to sodium current block may be related to the platforms and experimental conditions used to determine IC50 values for block [potential difference in properties of overexpressed sodium channels in HEK/CHO cells vs. native myocytes, experimental conditions (room temperature, low extracellular sodium conditions), voltage protocols used (often no consideration of voltage or rate dependence), and bath concentrations (with potential differences between achieved and nominal exposures)]. It is also possible that the retrospective analysis of clinical response may have been biased toward lower drug exposures. Despite these concerns, and based on present practices for evaluating drug effects on

sodium current, minimal sodium current block appears to be sufficient to slow cardiac conduction in normal myocardium.

Advances in cardiac monitoring in early clinical trials and automated ECG analysis have made it easier to interrogate effects of drugs on ventricular conduction (QRS duration) in early clinical studies. Given that preclinical data suggests a potential clinical liability for QRS prolongation with a promising clinical candidate, it should be possible to de-risk the candidate by directly evaluating clinical responses early in clinical development (as in early clinical dose-ascending phase one tolerability studies). Such efforts require consideration of the power of clinical studies to reliably detect small changes in the QRS interval.

6 L-Type Calcium Current

In working ventricular myocardium, the predominant inward current that flows after the action potential upstroke is L-type calcium current (Cav1.2, encoded by the CaCNA1C gene). The cardiac L-type calcium current was so named because of its slow kinetics of current decay ("L for long-lasting"); it has also been referred to as the dihydropyridine receptor due to its sensitivity to this chemical series (see below). In cardiac membranes, this voltage-dependent channel is composed of an alpha 1c subunit, b2a subunit, and a2-delta subunit; the alpha 1c subunit consists of the voltage sensor and channel pore composed of 4 homologous motifs, each containing six transmembrane segments. The auxiliary subunits play important regulatory roles that may be overlooked when using heterologous expression systems. It is likely that various functionally distinct subpopulations of L-type calcium channels with regionally distinct functional properties and regulation exist [e.g., those at dyadic junctions vs. plasma membrane microdomains such as lipid rafts and caveolae (see review by Best and Kamp 2012)].

The three classes of calcium channel pore alpha1 subunits (Cav.1, Cav2, and Cav3) demonstrate marked differences in their regulation: the Cav1 family is regulated primarily by second messenger activated kinase pathway and protein phosphorylation, while the Cav2 family is regulated by direct binding of signaling proteins; less is known about regulation of the Cav3 family. A second, smaller, and more rapid calcium current [termed T-type calcium current ("T" for transient), CaV3.x] is also present in embryonic heart and specialized tissues of adult heart [nodal regions and specialized conduction pathways; the reader is referred to a recent review for more details (Ono and Iijima 2010)].

L-Type calcium current plays critical roles in modulating multiple cardiac functions. Thus, the influx of calcium ions is responsible for initiating muscle contraction and modulating contractility. From an electrophysiological perspective, L-type calcium current plays a prominent role in defining the configuration of the ventricular action potential. In contrast to fast inward sodium current, the relatively slow kinetics of activation delays the contribution of this current until inactivation of the fast inward sodium current and the early part of the action potential plateau.

Under voltage-clamp conditions, ICa,L is activated at potentials positive to −40 mV, showing peak activation near +10 to +20 mV. This current also acts to sustain the action potential plateau at sufficiently positive potentials to enable activation of delayed rectifier current (hERG) that contributes to terminal repolarization (phase 3 of the action potential). ICa.L inactivation is complex, being dependent on both voltage and current [calcium-dependent inactivation (CDI); see Grandi et al. 2010; Tuckwell 2012 for reviews].

In regard to cardiac contractility and inotropy, the L-type calcium channel provides the transient influx of calcium ions responsible for calcium-induced calcium release (CICR) from sarcoplasmic reticulum stores, primarily through activation of the ryanodine receptors (RYR2) (see Bers 2002 for a review). As a consequence, intracellular calcium levels rise from low basal levels (<100 nM) to low micromolar levels with each action potential, leading to optimal binding of Ca^{++} to troponin C and induction of contraction. Thus, along with the sodium–calcium exchanger, L-type calcium currents figure prominently in modulating basal and transient levels of intracellular calcium to define intrinsic cardiac contractility and electrophysiology (see Banyasz et al. 2012). L-Type calcium current also demonstrates calcium-induced inactivation, a fact that highlights the integrated nature of cellular calcium handling and electromechanical coupling (an aspect of electrophysiological response often overlooked in studies employing channels expressed in heterologous systems). Finally, intracellular calcium levels likely also play signaling roles in the myocardium to influence more longer-term adaptation, such as hypertrophy; this area (important for longer-term chronic effects) is under active investigation.

L-Type calcium current plays a role in certain forms of arrhythmias. An early after depolarization (EAD) is a form of triggered electrical activity in which there is a slowing or reversal of repolarization during the plateau or early phase of action potential repolarization (see January and Riddle 1989; also Weiss et al. 2010). This premature depolarization results from a regenerative increase in net outward current, typically in the setting of slowed repolarization, indicative of reduced "repolarization reserve" (for a recent review, see Varró and Baczkó (2011)). In the voltage range of approximately −30–0 mV, there is overlap of steady-state activation and inactivation of ICa,L that results in the so-called "L-type window" current. Time- and voltage-dependent recovery from inactivation may allow a regenerative increase in ICa,L to elicit an EAD. If sufficiently strong (and properly coupled to surrounding myocardium), EADs may provide sufficient stimulus to produce a triggered beat (extrasystole) or run or triggered beats (potentially ventricular tachycardia). Block of L-type calcium current mitigates EAD activity. However, this effect is complex, in that other calcium-dependent currents (e.g., calcium current exchanger) may also be affected (see Banyasz et al. 2012). EADs represent an integrated cellular response that would not be detected in typical simpler (in vitro) ionic current studies. However, such activity has been reported in human pluripotent stem cell-derived cardiocytes [representing earlier in vitro studies not requiring animal tissues (Ma et al. 2011)]; such preparations may be amenable to earlier electrophysiological safety pharmacology studies derived from normal (Jonsson et al. 2012) or diseased sources (Liang et al. 2013).

Some hERG-blocking drugs are not linked to proarrhythmia (e.g., verapamil and fluoxetine). For these drugs, it has been postulated that hERG block may be mitigated by L-type calcium channel block, reestablishing a "balance" between inward and outward currents and thereby reducing proarrhythmia liability (Fermini and Fossa 2003; Martin et al. 2004). Indeed, recent computer simulations suggest that consideration of drug effects on three cardiac currents (hERG, INa, and ICa,L) improved the predictive classification of 31 marketed drugs (based on the assessment of AP prolongation in model correlated with clinical risk of torsades de pointes arrhythmia (Mirams et al. 2011)). It is also possible that the mitigation of proarrhythmic risk (associated with delayed repolarization) by L-type calcium channel block results from effects on calcium handling/homeostasis originally perturbed by electrophysiological changes (prolongation) of the action potential. In favor of this notion, a recent study by Johnson et al. (2013) using canine ventricular myocytes suggested that late diastolic release of sarcoplasmic calcium during beta-adrenergic stimulation caused prolongation of the following action potential by reducing calcium-dependent inactivation of L-type calcium current. The resulting prolongation of the action potential leads to increased beat to beat variability of repolarization, a recognized hallmark of proarrhythmia. Future studies will be necessary to properly attribute the contributions of direct channel effects vs. intracellular calcium handling effects to mitigation of proarrhythmia by hERG-blocking drugs. Such endeavors are important to prevent unwarranted elimination of evolving drug candidates from further consideration based solely on early detection of hERG blockade.

Numerous studies have demonstrated that ICa,L is modulated by drugs and other factors, a fact not unexpected considering the multiple functional roles for this current. A voltage-clamp study demonstrating the dynamic modulation of direct block of ICa,L with verapamil by state-dependent changes in the channel is provided by Nawrath and Wegener (1997). Other indirect effects of pharmacologic agents are recognized, for example, that beta-adrenergic stimulation increases inotropy (in part) by phosphorylation of L-type calcium channels through cAMP-dependent protein kinase A signaling (McDonald et al. 1994) to increase current (without affecting single channel density) as part of an integrated response to increase cardiac inotropy. Hypoxia also inhibits basal L-type calcium current, consistent with modulation of current by redox status of the cardiac myocyte (Hool et al. 2005). The calcium-sensing protein calmodulin also acts as a transducer and targets L-type calcium channel [see Saucerman and Bers (2012) for a recent review]. The role which drugs may play in modulating known physiologic alterations of channel function is largely unstudied and would not be detected in most early (non-cell-based) safety screening assays.

Calcium channel blockers act to directly reduce L-type calcium current. When considered using schemes proposed for antiarrhythmic drugs, they are placed into the Vaughan Williams Class IV category. In general, calcium channel blockers are conveniently characterized within three structural chemical groups, with each binding to different binding sites on the channel: phenylalkylamines (e.g., verapamil) bind to the V binding site, benzothiazepines (e.g., diltiazem) bind to the D

binding site, and dihydropyridines (e.g., nifedipine) bind to the N binding site. As this channel is present in the heart as well as smooth muscle layers of peripheral vasculature, calcium channel blockers may also dilate coronary arteries and peripheral arterioles in addition to reducing cardiac contractility (negative inotropic effect) and affecting conduction through the AV node (negative dromotropic effect). In general, dihydropyridines have minimal effect on cardiac conduction or heart rate, while verapamil and diltiazem are known for slowing of AV conduction and decreased SA nodal automaticity. In contrast to block of hERG and fast inward sodium current, translating the in vitro effects of calcium current reduction to either negative inotropic or chronotropic effects is more difficult due to the more integrated nature of these responses.

Despite the complex role that ICa,L plays in cardiac (and extracardiac) tissues, early functional screening (using voltage-clamp techniques) is valuable in the early detection of potential cardiovascular liabilities (hazard identification). However, the translation of macroscopic current data to inform on safety margins for clinical studies (risk assessment) requires an understanding of the role of ICa,L in more complex physiological systems. Such information may be provided by follow-up in vitro (tissues and organs) and in vivo (whole animal) electrophysiological studies that place early current studies in context. It should also be recognized that the knowledge of the regulation of normal complex physiological systems may be limited and less may be known for human disease states. Thus, care is necessary in attempting to translate such early signals to clinical effects, and attempts to overinterpret early safety margins should be avoided. While it is possible to monitor negative dromotropic effects (slowed PR conduction) of evolving drug candidates early in phase 1 clinical studies (e.g., via careful monitoring of PR interval changes on ECG linked to drug plasma concentrations), it is much more difficult to noninvasively assess acute drug effects on cardiac contractility.

7 Conclusions

One important role of the safety pharmacologist is to recognize the strengths and limitations of early exploratory studies in the setting of interpretation and translation to more integrated preclinical assays and final clinical studies. Such efforts will ultimately require comparing data across multiple assays with multiple compounds, ideally across multiple groups in the precompetitive space. For any early in vitro safety pharmacology assay, the ever-present challenge is to strike the appropriate balance between assay sensitivity (ability to detect true positive preclinical results) and assay specificity (ability to detect true negative preclinical results) in relation to "gold-standard" clinical observations. A recent study comparing results from the in vitro hERG functional current assay to clinical findings of QT prolongation in thorough QT studies (Gintant 2011) provides one approach to qualitatively assess the utility and performance of early safety assays based on defining receiver operator curves. However, it is likely that (even with assay optimization) overall performance of early safety assays will not be fully predictive due to the lack of

systems complexity of such early assays relative to the integrated responses observed clinically (including cardiac repolarization, blood pressure, heart rate). Despite this limitation, even such "less than perfect" approaches will enhance the probability of success of advancing novel therapeutics and provide for the more efficient discovery and development of safe and effective drugs.

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Safety Pharmacology in Drug Discovery and Development

Bruce H. Morimoto, Erin Castelloe, and Anthony W. Fox

Contents

Abstract

Safety pharmacology is essential throughout the spectrum of drug discovery and development. Prior to first-in-human studies, safety pharmacology assays, tests, and models predict the clinical risk profile of a potential new drug. During clinical development, safety pharmacology can be used to explore—and potentially explain—both predicted and unpredicted side effects (e.g., adverse events, changes in vital signs, abnormal laboratory values) in order to refine the original clinical risk profile. This chapter will introduce the reader to safety pharmacology's role in translational medicine: the science of translating potential drugs' on- and off-target nonclinical properties to clinical consequences in order to select the best drug candidates to move into early clinical testing. Case studies will be used to illustrate the importance of safety pharmacology testing throughout all phases of drug development.

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Keywords
Drug discovery • Drug development • On-target • Off-target • Translational medicine • Risk assessment • Clinical risk profile • Decision matrix • Case studies

1 Introduction

Which would have been as speedy in your end
As all the poisonous potions in the world,
And saved the treacherous labour of your son.
—1 Henry IV, Act 5, scene 4, lines 54–56 (1623 folio).

Strategy The contribution of safety pharmacology to effective drug development has matured over the past 10 years. Safety pharmacology is now its own acknowledged discipline with the central aim of predicting whether, and if so, then what, drug intolerability should be watched for when humans are exposed to an investigational drug (i.e., new chemical entity or NCE).

Moreover, in the interest of efficient drug development (especially in an era of diminishing resources), there is a need for facilitated "drug Darwinism," i.e., killing the potential losers sooner so as to allow finite resources to be redirected to better potential prospects. Increasing pressure to reduce the number of late-stage failures puts a premium on safety pharmacology studies that can further triage drug candidates as early as possible, preferably during the drug discovery phase, thus allowing (appropriate allocation of) finite resources to be directed to characterize the efficacy and safety of possibly better drug candidates (see Ringel et al. (2013) for commentary).

This chapter will argue that safety pharmacology should be a rigorous test phase, with intelligent study design and thorough data interpretation, prior to phase I clinical development. Safety pharmacology is not a box-checking exercise, involving conduct of a few stereotypical studies with brief drug exposures and the blinkered aim of mere sufficiency to avoid an IND or CTA clinical hold.

Much of the philosophy of safety pharmacology is drawn from the discipline of toxicology (Pugsley et al. 2008). Vehicle (i.e., placebo) and active controls and dose–response (range finding) exploration are commonly seen in these types of study. Both types of study are conducted according to *Good Laboratory Practice* (GLP) standards as per the ICHS7A and S7B guidance documents. GLP insures that a quality system of management controls is used in study conduct so that consistency, uniformity, reliability, and reproducibility are applied to the conduct of these nonclinical safety tests (see 21CFR58 and OECD 1998). However, the two disciplines differ in that:

- Safety pharmacology studies are conducted to explore both "on-target" and potential "off-target" adverse pharmacological actions of the NCE. This distinction is especially important during drug discovery since conduct of, and

discussions with, the medicinal chemists is most optimal to suggest changes to an existing compound so as to minimize "off-target adverse effects"; meanwhile, with the exception of drugs for malignancy, "on-target adverse actions" may cause the entire therapeutic approach (i.e., target) to be questioned.
- Safety pharmacology studies can be used to investigate specific, well-characterized mechanisms of drug toxicity (e.g., potassium channel blockade as a specific pharmacological property, compared with undifferentiated cardiovascular collapse that might be observed in an ordinary toxicology study). Note that from experience, the design of the safety pharmacology studies becomes more straightforward when the pharmacological properties of the NCE are well understood.

While safety pharmacology study designs are highly refined, they nonetheless originated from general pharmacology laboratories (Bass et al. 2004). The concepts of efficacy pharmacology (e.g., quantifying the concentration–response relationship accurately) also apply. However, the range of methods available for use in any given safety pharmacology facility will usually be narrower and much more rigorously validated than in a typical exploratory pharmacology laboratory. The difference is that "ordinary" efficacy pharmacology usually aims only to provide a mechanistic rationale for what may turn out to be a therapeutic (or toxic) effect. In contrast, safety pharmacology (per ICH S7A) is about how to avoid hazard to humans due to undesirable or unwanted pharmacological action(s). Therefore, since they contribute to delimiting clinical hazard, regulatory safety pharmacology studies are often conducted under GLP conditions, unlike almost all "ordinary" efficacy pharmacology studies. Moreover, the GLP requirement for rigorous protocols and validation of all equipment used (with documentation) provide reassurance of the integrity of the data, for the direct benefit of the human volunteers and patients who will subsequently be exposed to the drug. Thus, post-study quality assurance and audit are routine in safety pharmacology studies, unlike "ordinary" efficacy pharmacology experiments.

Above all, risk assessment is central to the training and outlook of a safety pharmacologist, but not his/her exploratory pharmacologist colleague.

2 Safety Pharmacology in Drug Discovery: Selecting the Best Drug Candidates

> Begin with the end in mind. . .
> Stephen Covey

Tactics Safety pharmacology studies may be incorporated into drug discovery (i.e., termed "frontloading" studies) to help in the selection of compounds with the most optimal pharmacological properties. Early drug discovery begins with a

target product profile (TPP), which defines the optimal and/or acceptable properties of the potential new drug (see the FDA guidance document Target Product Profile—A Strategic Development Process Tool, 2007 for details). The TPP is driven by the physicochemical properties, therapeutic indication, regulatory constraints, patient population, and competitive situation, including relative properties compared with other drugs (if any) for the same indication. Safety pharmacology can play a key role in defining, evaluating, and refining this TPP.

Compounds in early-stage, discovery-phase development come from a variety of sources. Characterization of the response to the compound is one of the goals or objectives of safety pharmacology during these early stages (Fig. 1). A compound initially binds to its target (such as an enzyme or a receptor). This drug-target complex might then be capable of eliciting or blocking a pharmacological response, such as a change in ion current across the cell membrane, generation of a second messenger, or activation of DNA transcription or one of the multiple other potential post-target translational processes. Usually, these pharmacological responses can be measured in vitro or using cell-based assays and may require a few milligrams of compound (Whitebread et al., 2005). The observed pharmacological responses might include muscle contraction, paralysis, or secretion of a hormone. Such responses can be ranked in order of complexity, from biochemical at the cell level to changes in an organ or tissue to physiological or behavioral changes in the whole organism. As response complexity increases, the number of potential signal inputs also increases, and thus experimental controls become more numerous and sophisticated. The amount of compound required also increases with assay complexity. In situ (e.g., isolated Langendorff heart) and in vivo (e.g., anesthetized guinea pig), studies frequently require several grams of drug to be available.

Putative Ligand Screens Compound selectivity is initially assessed by screening a putative ligand against a wide variety of molecular targets (i.e., receptors, ion channels, enzymes, and transporters). These ligand displacement assays are described elsewhere in the book (see chapter by Gintant for detailed review).

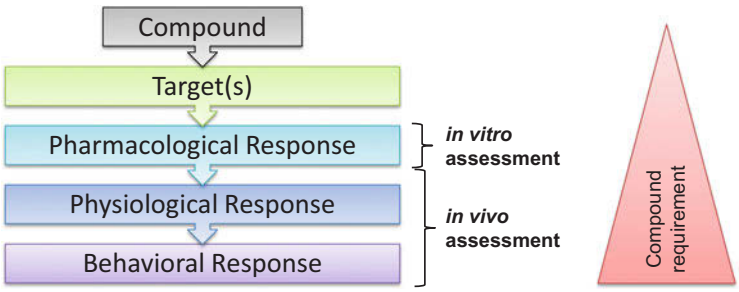

Fig. 1 Characterization of a compound's effect. A compound often binds to a particular target or targets (e.g., a receptor or an enzyme) and elicits a pharmacological response like the activation of a signal transduction pathway. This in turn results in a physiological change, such as a change in heart rate or insulin secretion. These physiological changes can then result in a change in the organism's behavior

There are a number of contract service providers, such as Eurofins Panlabs, Cerep, and PerkinElmer–NovaScreen, that offer binding assays for numerous molecular targets (e.g., Cerep offers a panel of over 630 validated in vitro pharmacological assays that cover a broad range of targets). Usually, ~1 mg of compound is sufficient to screen against 120 or more potential targets using in vitro assays. "Hits" are initially identified by >50 % target occupation using a 0.1–1 μM concentration of the test compound. Thereafter, if needed, specific safety pharmacology studies to quantify potency (e.g., IC_{50}) of the (hopefully) small number of "hits" can follow. Note: this initial screening procedure (binding assay) does not distinguish between agonists and antagonists.

Target-Directed Effect Assessment The ability of a compound to interact with an unintended target is referred to as an "off-target" effect. Although compounds are designed to activate or inhibit a particular target, be it a receptor or enzyme, they are seldom 100 % selective for that intended target. This lack of selectivity is much more likely for small chemical molecules (NCE) than for monoclonal antibodies or other large, biological drugs. There are times when the quantitative comparison of "on-target" and "off-target" effects becomes critical in decision-making, and this is, again, within the realm of safety pharmacology. For example, if an unexpected pharmacological response for a lead molecule is observed in a toxicity study or clinical trial and is related to off-target activities, it may be possible to switch to a backup compound that happens to have less of the off-target interactions. Failing that, conducting additional medicinal chemistry on the compound so as to "engineer out" the off-target activity (Fig. 2) might yet salvage the NCE pharmacophore as of potential therapeutic value. However, if the undesired pharmacology is an

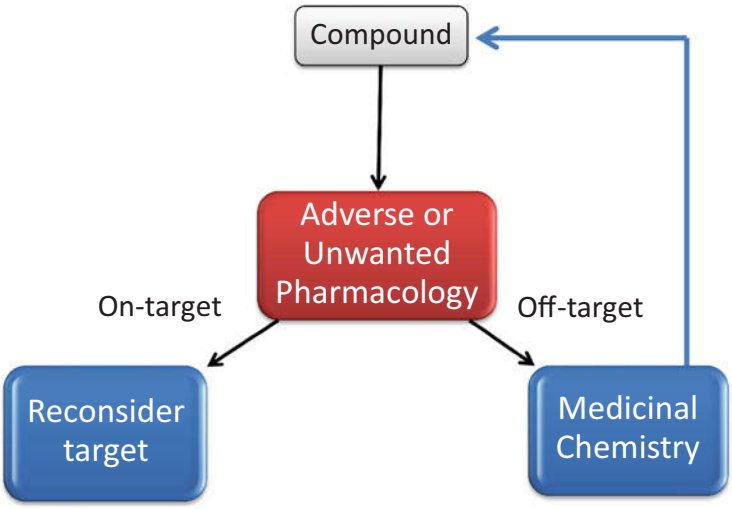

Fig. 2 Importance of distinguishing on- versus off-target pharmacology. The definition of "adverse" will depend on the target profile including an assessment of the relative safety margin. Medicinal chemistry can be used to improve selectivity of the compound

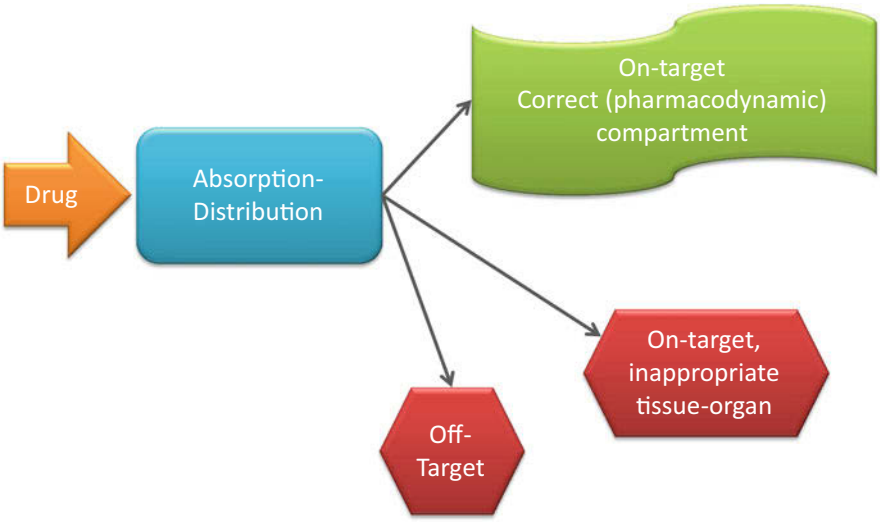

Fig. 3 Tissue–organ distribution in adverse pharmacology. On-target pharmacology can result in adverse effects if engagement of the target is in the wrong or inappropriate tissue

adverse on-target effect, then serious consideration must be given to whether that target is appropriate for therapeutic intervention at all.

Adverse, on-target interactions can also occur when a candidate drug interacts with the appropriate receptor(s), but in unintended tissues or organs (Fig. 3, and see below). Occasionally, the adverse on-target pharmacological property can be remedied by designing a partial agonist/antagonist or designing some sort of allosteric regulator of the target (Li et al. 2014). Occasionally, pharmacokinetic variations or changing the route of administration, such as subcutaneous or intramuscular routes of administration for an IV drug, can avoid Type A (concentration-related) adverse events without sacrificing exposure (measured as area under the time–concentration curve). Localized administration, direct to the target organ, or finding a backup molecule with better tissue selectivity can also minimize on-target toxicity. The utility of such modifications to the candidate drug or its route of administration can be confirmed in safety pharmacology studies.

Safety Pharmacology Throughout Drug Development Safety pharmacology studies rarely conclude when a compound has passed the first-in-human (FIH) study stage. The receptors, enzymes, and ion channels (etc.) of interest for "off-target" interactions are defined not only by the TPP but also by adverse events that arise in toxicology as well as early clinical studies (Table 1; Bowes et al. 2012). Receptors, ion channels, or enzymes identified in safety pharmacology studies that are associated with potential significant clinical hazard are kept under strict review and may be investigated with further complementary in vitro safety pharmacology studies. These types of targets are often then referred to as "high-impact targets."

For example, the cardiac potassium channel Kv11.1 (that carries the repolarizing current) is strongly associated with the potential for a cardiovascular hazard (i.e., QT prolongation and torsades de pointes) and is included in almost all small molecule development plans [see Lindgren et al. (2008), for a survey of industry practice]. Other high-impact targets may only be revealed during phase II or phase III clinical experience, especially when they are associated with an underlying disease state that cannot be studied in a normal volunteer (e.g., a compound with emergent negative inotropism after exposure to a patient with class I congestive heart failure). Low-impact targets, i.e., those with little or no adverse pharmacology associated with them, such as binding to the melanin receptor, are easily overlooked during early safety pharmacology profiling and yet might suggest, for example, that detailed intraocular examinations should be included in clinical trials to assess the retinal melanocytes for pre-malignant or malignant transformation.

Table 1 provides a partial list of safety pharmacology targets commonly used for initial profiling of "lead" drug candidates. The receptor targets include those of major neurotransmitter systems like adenosine, adrenaline, dopamine, histamine, serotonin, GABA, and acetylcholine (nicotinic and muscarinic). Muscarinic receptors are a good example of the potential for adverse pharmacology associated with development of an NCE. The M1 subtype has been associated with cognitive and gastric effects, whereas both the M1 and M3 subtypes can result in constipation, vision problems, and dry mouth; therefore, avoidance of off-target interactions is warranted. Ion channels are also critical targets to evaluate as they control a variety of cellular processes.

The International Union of Basic and Clinical Pharmacology (IUPHAR) hosts a public database of receptors, ion channels, nuclear hormone receptors, and enzymes (www.iuphar-db.org). Similarly, the British Pharmacology Society (BPS) has a complementary guide to receptors and channels database (http://guidetopharmacology.org/) that can be reviewed as well. Thus, each contains a wealth of information that relates target types with potential pharmacological responses, archetypal ligands, and hence helps predict drug intolerability in the clinic. Likewise, some targets are also known to be "promiscuous," meaning many chemical structures can interact with the target (e.g., the hERG potassium channel).

Clinical Correlates An understanding of the pharmacological and physiological response for a particular target is helpful in predicting potential outcomes. For example, if a compound has off-target interactions with angiotensin, endothelin, or α-adrenergic receptors, dose-dependent changes in blood pressure might be observed during conduct of in vivo studies. Off-target interactions with dopamine D_2 receptors can result in CNS side effects that include dyskinesia, erectile dysfunction, or neurodegeneration. These "off-target" interactions can be explored in vivo or they can be part of the potential pharmacological profile of the compound in human clinical studies. Sometimes, these off-target interactions can be monitored

Table 1 Common receptors and enzymes for off-target evaluation

Target	Major organ system	Effect(s)
Acetylcholinesterase	CNS, CV, GI, pulmonary	Salivation, GI motility, muscle paralysis, respiratory arrest, CV collapse
Adenosine A_1 receptor	CNS, CV	Bradycardia, AV block, renal vasoconstriction
Adenosine A_{2A} receptor	CNS, CV	Hypotension, heart rate changes, locomotor activity, platelet aggregation
Adrenergic α_{1A} receptor	CNS, CV, GI	Smooth muscle tone, hypertension
Adrenergic α_{2A} receptor	CNS, CV	Hypertension, hyperglycemia
Adrenergic β_1 receptor	CV, GI	Ventricular fibrillation, bronchospasm
Adrenergic β_2 receptor	Pulmonary, CV	Bronchodilation, bronchospasm
Androgen receptor	Endocrine	Carcinomas, hostility
Bradykinin receptor (B_1–B_2)	CNS, PNS	Cough, inflammation, vasodilation
Calcium channel (L-type)	CNS, CV	Hypotension
CB_1-cannabinoid receptor	CNS	Euphoria, dysphoria, anxiety, hypothermia
CB_2-cannabinoid receptor	Immune	Inflammation, decrease in bone mass
Cholecystokinin A receptor	GI	Increase in GI motility, gall bladder function
Cyclooxygenase 1 (COX1)	GI, pulmonary, renal	Bleeding, dyspepsia, renal dysfunction
Cyclooxygenase 2 (COX2)	Immune, CV	Anti-inflammatory, anti-mitogenic, hypertension
Dopamine D_1 receptor	CNS, CV	Dyskinesia, arousal, locomotor activation, vasodilatation, hypotension
Dopamine D_2 receptor	CNS, CV, endocrine	Decrease heart rate, syncope, hallucination, confusion, drowsiness, emesis, orthostatic hypotension, GI motility
Dopamine transporter	CNS	Addictive psychostimulation, depression, seizures, dystonia, dyskinesia, acne
Endothelin ET_A receptor	CV, development	Vasoconstriction, positive inotropy, cell proliferation
$GABA_A$ receptor	CNS	Muscle relaxation, ataxia, abuse potential, sedation, dizziness, depression
Glucocorticoid receptor	Endocrine, immune	Immunosuppression, osteoporosis
Glutamate NMDA receptor	CNS	Psychosis, hallucinations, delirium, seizures
Histamine H_1 receptor	CV, immune	Decrease in blood pressure, teratogenicity
Histamine H_2 receptor	CV, GI	Increase in gastric acid secretion, emesis, positive inotropy

(continued)

Table 1 (continued)

Target	Major organ system	Effect(s)
Lymphocyte-specific tyrosine kinase (Lck)	Immune	T-cell activation
Monoamine oxidase A (MOA)	CNS, CV	Drug–drug interaction potential, dizziness, nausea, sleep disturbances
Muscarinic M_1 receptor	CNS, CV, GI	Vagal effects, blood pressure changes, decrease gastric acid secretion
Muscarinic M_2 receptor	CV	Tachycardia, vagal changes, blood pressure changes
Muscarinic M_3 receptor	GI, pulmonary	Vagal effects, blood pressure changes, salivation, dry mouth
Neuropeptide Y	GI	Vasoconstriction, gut motility, gastric emptying
Nicotinic acetylcholine receptor	CNS, CV, GI	Nausea, sweating, tremor, bronchial secretion
Noradrenaline transporter	CNS, CV	Increase heart rate, blood pressure, locomotor activity, constipation, abuse potential
δ-Opioid receptor	CNS, CV	Analgesia, dysphoria, psychomimetic effects, convulsion, blood pressure
κ-Opioid receptor	CNS, CV, GI	Decrease GI motility, increase urinary output, sedation, dysphoria, confusion, dizziness, decrease locomotion, tachycardia
μ-Opioid receptor	CNS, CV, GI	Sedation, decrease GI motility, pupil constriction, abuse liability, respiratory depression, hypothermia
Phosphodiesterase 3A	CV	Increase heart rate, decrease blood pressure, thrombocytopenia
Phosphodiesterase 4D	CNS, immune	Anti-inflammatory, emesis, vasculitis
Potassium channel hERG	CV	QT prolongation
Potassium channel KCNQ1	CV	Hypotension, hypoglycemia
Serotonin 5-HT_{1A} receptor	CNS, endocrine	Decrease in body temperature, reduced REM sleep
Serotonin 5-HT_{1B} receptor	CNS, CV	Cerebral and coronary artery constriction, increase in blood pressure
Serotonin 5-HT_{2A} receptor	CNS, CV	Platelet aggregation, memory impairment, hallucinations, serotonin syndrome
Serotonin 5-HT_{2B} receptor	CV, pulmonary	Cardiac valvulopathy, pulmonary hypertension
Serotonin 5-HT_3 receptor	GI, endocrine	Emesis, gastric emptying, hyperglycemia, constipation, dizziness
Serotonin transporter	CNS, CV	Increase GI motility, decrease upper GI transit, insomnia, nausea, sexual dysfunction
Sodium channel	CV	Prolonged QRS interval, slowed cardiac conduction

(continued)

Table 1 (continued)

Target	Major organ system	Effect(s)
Vasopressin V_{1A} receptor	Renal, CV	Water retention, increase in blood pressure, decrease heart rate, cardiac hypertrophy

and provide an indication of pharmacological action and adverse events to watch out for in the clinical program.

On-target-mediated adverse pharmacology can result in an exaggerated pharmacological response. Monoclonal antibodies are commonly associated with these responses, for example, TGN1412 developed by TeGenero, which resulted in catastrophic systemic organ failure (Stebbings et al. 2009).

Alternatively, a particular target for therapeutic intervention likely has more than one physiological function. Consider Fig. 1 in which a single target then has multiple pharmacological, physiological, and behavioral responses. Moreover, the intended target may also be expressed in other tissues or organs (Fig. 3), and "on-target" interactions in the unintended tissue may lead to an adverse pharmacological profile.

Gamma secretase (GS) is a good example of the potential for on-target, improper tissue pharmacological activity with clinical correlates and hence adverse drug effects. Alzheimer's disease (AD) is characterized by abnormal deposits of beta-amyloid peptide (Abeta) in the brain. GS is the enzyme that synthesizes Abeta from a substrate known as amyloid precursor protein (APP). Therefore, one hypothesis for development of a therapeutic drug is that if Abeta synthesis can be reduced by inhibiting GS, then this could be beneficial to early-stage AD patients. However, APP in the central nervous system is not the only substrate for GS. GS is also found in crypt cells of the small intestine, where its role is to catabolize a protein called NOTCH and aid normal gastrointestinal (GI) physiology. Thus, GI intolerability is seen in clinical trials of GS inhibitors, which is an on-target, inappropriate tissue enzyme inhibition in the gut instead of the brain. In this case, second-generation molecules are now being investigated with the aim of allosteric modulation to restrict their effects to the CNS (Grosveld 2009; Staal and Langerak 2008).

There are numerous other examples of on-target, inappropriate tissue pharmacology. Inhibition of the phosphodiesterase (PDE) enzymes, in particular the PDE4 isoenzyme, may be beneficial for asthma and chronic obstructive pulmonary disease (COPD). However, PDE4 inhibition also causes profound emesis, as do some dopaminergic agonists designed for Parkinson's disease. Similarly, the first generation of histamine receptor antagonists (as discussed below) brought with them antimuscarinic properties and caused sedation. Thus, right-target, wrong-tissue pharmacology can prevent a drug from reaching the market and/or result in discontinuation of drug development when drugs with a superior profile follow quickly after the target is validated for a particular therapeutic indication.

3 Safety Pharmacology in Drug Development: Translation to the Clinic

Primum non nocere. . .First, do no harm
Attributed to the Hippocratic Corpus, 4th century BC

Strategy When lead candidate and backup compounds are selected from drug discovery platforms to enter the prehuman drug development phase (so-called pre-Investigational New Drug (IND), IND-enabling, or FIH-enabling development), safety pharmacology principles derived from appropriate studies can further assist in estimation of the possible dose or concentration margins between wanted and unwanted effects of the compound. Prediction of safety pharmacology endpoints and potential adverse events in clinical trials then becomes important, even if there is an imperfect translation.

Valuable predictors of clinical hazard can be gleaned from all nonclinical studies of an NCE. While appropriate design of general pharmacology and toxicology studies may reduce the number of separate safety pharmacology studies required, intelligent general toxicology study design can make safety pharmacology studies much more informative. Thus, appropriate IND-enabling safety pharmacology studies—nonclinical pharmacology studies specifically designed ". . .to investigate the potential undesirable pharmacodynamic [PD] effects of a substance on physiological functions in relation to exposure. . ." (see ICH S7A 2012)—become pivotally important.

Safety pharmacology studies are ultimately tailored to the specific characteristics of an NCE and the clinical indication(s) for which the NCE is proposed. Therefore, ideally, an understanding of the NCE's clinical development plan (and its regulatory hurdles) should precede and guide safety pharmacology study selection for an NCE. Typically, this takes place as an evolution of the TPP, and the sooner it evolves, preferably using expert input, then the more likely that the relevant safety pharmacology studies will be selected with efficiency and economies of time and cost.

Like the safety pharmacology tests conducted during lead candidate selection in drug discovery, these later IND-enabling safety pharmacology studies should not be considered as a "box-checking" exercise. While early-stage safety pharmacology studies can be hypothesis generating for the future design of the GLP compliant studies, the IND-enabling studies should attempt to elucidate problems that could arise later in clinical development. Observations from discovery stage safety pharmacology studies could and should be followed up with studies will enhance understanding of the mechanism and the dose–response relationship (if any) for the observed effects. Both become important in selecting the initial clinical dose and the dose escalation scheme in the first-in-human phase 1 studies. Occasionally, such studies will even suggest a new and unexpected indication.

Tactics At this IND-enabling stage, key questions to consider include:

- Which patient population(s) may benefit from this NCE?
- What patient-population-specific challenges (e.g., age, kidney or liver function, reproductive potential, etc.) may impact safety pharmacology study selection for this NCE?
- What treatment duration will be required for patients to receive benefit from this NCE?
- What route or routes of administration are feasible for this NCE?
- Of the feasible routes of administration, which is planned for clinical trials?
- Given the preferred route or routes of administration, what administration-route-specific risks are anticipated in patients (e.g., injection site reactions for injectables, skin or mucosal irritation for topicals or transdermals, etc.)?
- Given the formulations available (or likely to be available) for the NCE, what formulation-specific risks are anticipated in patients (e.g., hyperosmolar injectables)?
- Given predicted or known pharmacodynamic (PD) characteristics of the NCE, what PD-specific risks are anticipated in patients?

The answers to such questions will clarify safety pharmacology study requirements, key protocol design elements, and the necessary timing of such trials relative to the clinical research program.

Unless scientifically irrelevant or irrational, the "Safety Pharmacology Core Battery" (see ICH S7A) must be completed, in a GLP-compliant manner, prior to initiation of the FIH study. "The purpose of the safety pharmacology core battery is to investigate the effects of the NCE on vital functions…The cardiovascular, respiratory, and central nervous systems are usually considered the vital organ systems that should be studied in the core battery." Provided these do not reveal undue hazard or risks to potential human subjects, first-in-human studies may be initiated. Key data elements must be incorporated into applications for clinical trial authorizations. In the USA, for example, safety pharmacology data would be included in Section 8 of the Investigational New Drug (IND) application (see 21CFR312.23) and in the Clinical Investigator Brochure (see ICH E6 Good Clinical Practice section related to CIB content and format). Ultimately, if the drug succeeds, these studies will appear in the harmonized electronic Common Technical Document (eCTD) which is the means by which the pharmaceutical industry information on a drug candidate can be transferred to or can be sent to the regulatory agency.

To assess human risk, the predominant adverse events reported in clinical trials are presented in Table 2. Metabolic and hematological changes can be evaluated with clinical chemistry endpoints in nonclinical toxicology studies. Likewise, pathological changes can also be assessed in toxicology studies. However, a vast majority of the adverse events observed in clinical trials would require specific pharmacological studies to identify. The major organ systems outlined as vital in function in the ICH S7A guidance include the CNS, cardiovascular, and

Table 2 Common clinical adverse effects by organ system

Neurological	Psychiatric	Endocrine	Renal
Headache	Delirium, confusion	Thyroid dysfunction	Nephritis
Dizziness	Depression	Sexual dysfunction	Nephrosis
Sleep disorders	Hallucination	Gynecomastia	Tubular necrosis
Tremor	Drowsiness	Galactorrhea	Renal dysfunction
Seizure	Sleep disturbance		Bladder dysfunction
Extrapyramidal			Nephrolithiasis

Gastrointestinal	Hematology	Dermatology	Cardiovascular
Nausea-vomiting	Agranulocytosis	Erythemas	Arrhythmias
Diarrhea	Hemolytic anemia	Urticaria	Hypotension
Constipation	Pancytopenia	Photodermatitis	Hypertension
Gastritis	Thrombocytopenia	Eczema	Congestive heart failure
Dry mouth	Megaloblastic anemia	Hyperpigmentation	Angina-chest pain
Pancreatitis	Clotting or bleeding	Acne	Pericarditis
Ulceration	Eosinophilia	Alopecia	Cardiomyopathy

Metabolic	Respiratory	Musculoskeletal	Ophthalmic-otological
Hyperglycemia	Airway obstruction	Myalgia–myopathy	Disturbed color vision
Hypoglycemia	Pulmonary infiltrates	Rhabdomyolysis	Cataract
Hyperkalemia	Pulmonary edema	Osteoporosis	Optic neuritis
Hypokalemia	Respiratory depression		Retinopathy
Metabolic acidosis	Nasal congestion		Glaucoma
Hyperuricemia			Corneal opacity
Hypouricemia			Deafness
			Vertigo
			Tinnitus

respiratory systems. Although not specifically delineated in the guidance, renal and GI safety pharmacology studies also warrant attention when attempting to translate nonclinical findings to the clinic. Some common adverse events like cramps, sweating, xerostomia, headache, dizziness, nausea, psychiatric disturbances, cough, and skin rashes are difficult, if not impossible, to evaluate in animals (Greaves et al. 2004; Fletcher 1978). As safety evaluation in animals is imperfect in toxicology studies, well-designed and well-executed safety pharmacology studies can be complimentary and provide insight into what one might expect in clinical studies.

All these observations and findings will be included in the Clinical Investigators' Brochure (CIB). Indeed, the initial guidance for clinical hazard potential may depend almost entirely on safety pharmacology findings because of an unspectacular toxicology profile, the lack of human exposure, and the fact that normal volunteers are unlikely to be capable of exhibiting certain adverse event types

(e.g., the cardiovascular reserve of typical phase I study normal volunteers far exceeds that of smokers over age 45 years with hypertension). The characterization of adverse events as expected or unexpected (21CFR312.32(a) and comparable regulations in Europe and Japan) can also crucially depend upon safety pharmacology findings. For example, a drug that is a demonstrated peripheral vasodilator in safety pharmacology studies might be *expected* to be associated with edema of the dependent parts of the body in clinical trials.

Caveat Much of the above is generalization and example. Automatic rules exist neither for how to translate nonclinical findings to the prediction of human safety risk nor how and when particular safety pharmacology studies can contribute to that translation. Judgment and experience can only guide risk assessment, and drug developers are well advised to listen when regulatory agencies or institutional review boards (or ethics committees) ask questions; they, too, are assessing the weight of evidence in an environment of great uncertainty prior to allowing human exposures. We offer the following case study to illustrate these general principles.

4 The Case of Two Antihistamines

(1) Right Target, Wrong Tissue Histamine-H1 receptor antagonists are used for the treatment of allergic rhinitis, atopic dermatitis, and urticaria. A general side effect of the older ("first-generation") H1 antagonists (e.g., diphenhydramine, promethazine, and doxylamine) was sedation. Sedation occurred because these older drugs crossed the blood–brain barrier (BBB) and interacted with brain H1 receptors (a classic example of an on-target, wrong tissue adverse event). Second-generation antihistamines, such as loratadine and cetirizine, were designed to not cross the BBB and are thus more selective for peripheral H1 receptors, as confirmed by various CNS safety pharmacology studies. These newer drugs have far fewer CNS side effects, are now referred to as "nonsedating antihistamines," and are even sold over the counter without a warning about driving or operating heavy machinery.

(2) Completely Off Target Another antihistamine, terfenadine, provides an example of how modern safety pharmacology and metabolism profiling in drug discovery could have prevented the drug from entering clinical development and avoided clinical hazard and an expensive product withdrawal. Terfenadine was marketed worldwide as the first nonsedating antihistamine and was approved in the USA in 1985. By 1990, an estimated 100 million patients were taking terfenadine for allergies. Exposure of this large patient population was also associated with reports of serious ventricular arrhythmias and QT interval prolongation, which had not been detected in the relatively small-scale phase III studies. In 1997, all products containing terfenadine were removed from the US market. At the time, hERG and cytochrome P450 (CYP) screening were not routine. It turned out

(possibly unexpectedly for those familiar with "first-generation" H1 antagonists) that terfenadine has high affinity to the hERG channel (a classic example of an off-target interaction driving an adverse event). The principal metabolite of terfenadine is fexofenadine (converted by cytochrome CYP450 3A4), which is also an HI antagonist, but fortunately was a much weaker inhibitor of hERG. Inhibition of terfenadine metabolism (e.g., competition for CYP 3A4 by another drug, such as erythromycin or eletriptan) can cause dangerously high plasma levels of unmetabolized terfenadine, leading to hERG blockade, ventricular tachyarrhythmias, and *torsades de pointes* (Monahan et al. 1990). Thereafter, interactions with the hERG channel then became a part of the "standard battery" of safety pharmacology testing for almost all drugs in development for clinical use (see ICH S7B and ICH E14).

The terfenadine experience provides the safety pharmacologist with several key priorities during lead candidate selection and early drug development:

1. Carefully evaluate off-target interactions with high-impact targets like hERG.
2. Understand drug metabolism and the potential for drug–drug interactions.
3. Develop an appreciation for active drug metabolites.
4. Be alert for the unexpected and design safety pharmacology studies accordingly.

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Part II

The Safety Pharmacology Core Battery

CNS Adverse Effects: From Functional Observation Battery/Irwin Tests to Electrophysiology

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Abstract

This chapter describes various approaches for the preclinical assessment of drug-induced central nervous system (CNS) adverse effects. Traditionally, methods to evaluate CNS effects have consisted of observing and scoring behavioral responses of animals after drug is administered. Among several behavioral testing paradigms, the Irwin and the functional observational battery (FOB) are the most commonly used assays for the assessment of CNS effects. The Irwin and FOB are considered good first-tier assays to satisfy the ICH S7A guidance for the preclinical evaluation of new chemical entities (NCE) intended for humans. However, experts have expressed concern about the subjectivity and lack of quantitation that is derived from behavioral testing. More importantly, it is difficult to gain insight into potential mechanisms of toxicity by assessing behavioral outcomes. As a complement to behavioral testing, we propose using electrophysiology-based assays, both in vivo and in vitro, such as electroencephalograms and brain slice field-potential recordings. To better illustrate these approaches, we discuss the implementation of electrophysiology-based techniques in drug-induced assessment of seizure risk, sleep disruption, and cognitive impairment.

Keywords

Central nervous system • CNS • Irwin • FOB • Electroencephalogram • EEG • Brain slice • Field potential • Hippocampus • Seizure • Preclinical • Nonclinical • Rat • Mouse • Sleep • Cognition • Behavior

1 Introduction

Central nervous system (CNS) adverse effects have accounted for approximately 10 % of the 121 marketed pharmaceuticals withdrawn from sale over the period of 1960–1999 (Fung et al. 2001). This value has been contextualized by others noting that by comparison only 7.5 % of drug withdrawals during the same period were attributed to the cardiac arrhythmia known as torsades de pointes (Fung et al. 2001; Redfern et al. 2005). For all compounds evaluated by Fung et al. (2001), the most common CNS-related reasons for drug withdrawals included neurologic (4.1 %), psychiatric (3.7 %), and abuse liabilities (3.7 %). By convention, new candidate drugs are first screened for potential adverse CNS effects by monitoring behavioral changes in preclinical studies (Greaves et al. 2004). There are a number of behavioral assays available to assess the general effects of compounds on the CNS using an initial broad-based screen, and among these, the most common are the Irwin assay (Irwin 1968) and the functional observational battery (FOB) (Moser et al. 1995). These assays are widely used by safety pharmacology groups across the pharmaceutical industry and are recognized by the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for

Human Use and Safety Pharmacology Studies for Human Pharmaceuticals S7A (ICH S7A) as important tools for assessing the safety of new chemical entities (Guidance for industry: S7A safety pharmacology studies for human pharmaceuticals. US department of health and human services food and drug administration center for drug evaluation and research (CDER) center for biologics evaluation and research (CBER) 2001; M3(R2) nonclinical safety studies for the conduct of human clinical trials and marketing authorization for pharmaceuticals. US department of health and human services food and drug administration center for drug evaluation and research (CDER) center for biologics evaluation and research (CBER) 2010; US Environmental Protection Agency 1998). The objectives of these tests as described in ICH S7A are to: (1) identify undesirable pharmacodynamic properties with relevance to human safety, (2) further evaluate effects seen in toxicology or clinical studies, and (3) investigate possible mechanism of action (Guidance for industry: S7A safety pharmacology studies for human pharmaceuticals. US department of health and human services food and drug administration center for drug evaluation and research (CDER) center for biologics evaluation and research (CBER) 2001).

Unlike functional assays for cardiovascular and respiratory organ systems which use quantitative and objective measurements, assessment of CNS function using behavioral assays is largely dependent on multiple subjective endpoints. While recognizing the benefits of an integrated behavioral assessment (Moser and MacPhail 1992; MacPhail et al. 1997; Moser et al. 1997a, b), we would argue that these tests alone are not sufficient to understand the mechanisms associated with many CNS adverse effects. As is the case with all areas in drug-safety assessment, there is an expectation that progress in the evaluation of drug-induced CNS effects will result in earlier testing, more quantitative and physiologically relevant endpoints, higher throughput, as well as better assay sensitivity and specificity with an overall improved predictivity of clinical outcomes. The challenge remains to be able to integrate drug-induced behavioral responses with more direct measures of changes in the neuronal circuits responsible for such behaviors. In this chapter we review various approaches to assess CNS adverse effects, starting with traditional behavioral assays such as Irwin and FOB followed by more quantitative techniques such as in vivo and in vitro electrophysiology.

2 Behavioral Assessments: Historical and Regulatory Perspective

Behavioral assessments have long been recognized as a useful approach to gauge drug-induced CNS adverse effects of chemicals and pharmaceuticals (Irwin 1968; National Research Council 1984; Weiss and Laties 1979; Moser 1991). Historically, there have been marked differences in the test batteries used for evaluating new compounds in the pharmaceutical or chemical industries. In the 1960s, Samuel Irwin (1962, 1964, 1968) described a multi-parametric observational battery to assess the overall behavioral, neurological, and autonomic state of an animal that

was widely adopted for use within the pharmaceutical industry. The method entailed the systematic evaluation of nearly 50 parameters ranked using a detailed rating scale. The resulting profile could be used to differentiate between potential central or peripheral nervous system actions. In contrast to the Irwin test, the FOB arose from the need to improve cage-side observations made in the chemical industry, especially in relation to pesticides, to assess CNS adverse effects. In the past, such observations were only noted when clear adverse events were seen and a systematic elaboration of parameters was not available. In addition, detailed descriptions of the signs observed, including the magnitude and time course of effects, were lacking. Recommendations from experts (National Academy of Sciences (NAS) 1975; Brimblecombe 1979; Mitchell and Tilson 1982) focused concerns about inadequate neurotoxicity testing in the chemical industry. Consequently, the United States Environmental Protection Agency (EPA) developed guidelines for several behavioral tests including some loosely based on the Irwin screen (Sette 1989). The regulatory requirements for preclinical safety pharmacology testing were formalized by ICH S7A guidelines which describe a streamlined package geared to meet the minimal requirements of regulatory agencies (Guidance for industry: S7A safety pharmacology studies for human pharmaceuticals. US department of health and human services food and drug administration center for drug evaluation and research (CDER) center for biologics evaluation and research (CBER) 2001). The Irwin or modified Irwin tests (Irwin 1968) and the FOB (Moser 1991) remain the two most commonly used observational testing paradigms to assess drug-induced CNS adverse effects and are considered equivalent by ICH S7A guidelines. The Irwin and FOB tests can be conveniently applied to longitudinal study designs to assess dose range, duration, magnitude, and reversibility of behavior and physiological functions (Moser 1991, 2011). These behavioral tests are part of the safety pharmacology core battery studies performed in pharmaceutical industry to evaluate motor activity, behavioral changes, coordination, sensory/motor reflex responses, and body temperature.

2.1 Irwin Experimental Protocol

Since the original description, multiple permutations of the Irwin screen and FOB have been published with variations from numerous investigators (Redfern et al. 2005; Irwin 1968; Moser et al. 1995; Roux et al. 2004; Himmel 2008; Mattsson et al. 1996; McDaniel and Moser 1993). For the remainder of this chapter, we will refer to the Irwin assay although the general principles of the methodology and data interpretation are applicable to other behavioral assessments, including the FOB. A generic Irwin study protocol is shown in Fig. 1 along with a description of the study design.

Rats or mice of either sex can be used in the Irwin test. Various rat or mouse strains may be employed, but often it is necessary to revalidate assay parameters because some behavioral characteristics may differ between strains. For example, different rat strains that may differ in body weights may consequently display

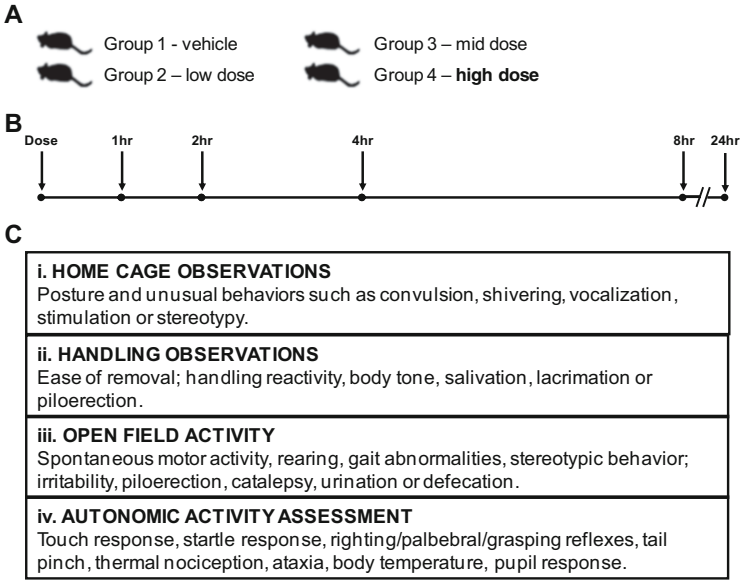

Fig. 1 *Study design for the preclinical assessment of potential CNS liabilities*. Multiple permutations of observational behavioral tests have been published, and study protocols may also be modified depending on the specific pharmacokinetic or expected behavioral effects of the test compound. This example illustrates a generic Irwin study protocol typical of those used within preclinical CNS safety pharmacology studies; a detailed description of the study design is included in the main text. (**a**) *Pre-study*: prior to the start of the study, animals are acclimated, randomized, and distributed across test groups and inspected regularly to ensure that behaviors are uniform across each group. Clinical signs and body weight are also recorded. (**b**) *Study time course*: animals are dosed (time = 0) and placed back in the home cage, and each animal is then observed at discrete time points post-dose according to the pharmacokinetic properties of the test compound. Dosing is staggered such that one observer can record parameters from multiple animals. In this example, as indicated by each *arrow*, animals are observed at $t = 1$, 2, 4, 8, and 24 h post-dose. Following study termination, clinical signs and body weights are recorded. (**c**) *Observations recorded at each time point*: the order of testing should progress from the least to the most manipulative procedures, starting with home cage observations (i), followed by observations during handling (ii). The animal is then placed in a clear viewing chamber (open field), where a number of parameters are measured during a 3-min exploratory period (iii). Finally, a number of more invasive measures are made, including responses to various stimuli (i.v.). Following the measurement of body temperature, the animal is returned to the home cage. This entire test procedure is completed at each time point for the duration of the experimental time course. A skilled observer typically completes this battery of tests in approximately 5 min on each animal

different baseline locomotor activity (Roux et al. 2004). Adult animals are used in most studies, but, depending on the target patient population, it may be appropriate to use animals at different stages of development, i.e., neonatal, juvenile, adult, or pregnant animals. Note that the behavioral effects of test compounds may differ substantially throughout development (Himmel 2008). A minimum of 3 days acclimation in the test facility is recommended before starting experiments to minimize the stress from transportation which may have an impact on body weight,

heart rate, or activity levels (Capdevila et al. 2007). Likewise, it is recommended to avoid bedding and cage changes within 18 h of the start of the study (Roux et al. 2004).

Animals should be randomized and distributed evenly across treatment groups. Typically, a vehicle and three dose groups are used, and it may also be necessary to include a satellite group for blood sampling and another as a positive control group. Statistical power analysis helps determine an appropriate group size, and as a default, six animals per group are recommended.

Regulatory guidelines suggest testing multiple doses to explore the dose–response relationship. The main goal of the observational study is to determine the highest dose that can be tolerated without observable behavioral effects (the no observable effect level, NOEL) and to determine the active dose range to a level that causes a noticeable adverse event. With this in mind, where possible, dose levels should be selected based upon a prior animal study such as a maximum tolerated dose (MTD) toxicity assessment that informs the dose selection for the Irwin test. Ideally, the low dose would approximate the human therapeutic dose or a low multiple thereof, while the high dose should be sufficient to produce moderate behavioral and physiological effects. The intermediate dose would further characterize the dose–response relationship.

Rats or mice should receive test article via the clinically intended route of administration where feasible. Regardless of the route of administration, exposure to parent compound and major metabolites (>20 % parent) should be similar to or greater than that anticipated in humans (Guidance for industry: S7A safety pharmacology studies for human pharmaceuticals. US department of health and human services food and drug administration center for drug evaluation and research (CDER) center for biologics evaluation and research (CBER) 2001). The use of several routes of administration may be warranted in situations where marked differences in systemic or local exposure occur or when multiple routes of administration in human are anticipated (e.g., loading dose followed by a maintenance dose). The route of administration should be considered carefully as it can affect rate of adsorption, slope and variability of the dose–response curve, as well as intensity and duration of effect (Buxton 2006).

Ideally, observational time periods are defined based on the pharmacokinetic (PK) properties of the test article with at least one time point approximating the time at which the maximum plasma concentration (C_{max}) occurs. Subsequent time points typically span 24 h or longer depending on the duration of the drug's half life.

Testing is initiated with undisturbed observations taking place first in the home cage and then in an open arena. A manipulative phase follows with each animal exposed to increasingly more provoking stimuli. This process is repeated at multiple time points which are defined according to the PK profile of the test compound. A generic list of parameters measured and the order in which they are performed is shown in Table 1 and Fig. 1, respectively. The reader is referred to reports from multiple laboratories for additional lists of parameters, alternative scoring scales, and convenient data collection forms (Redfern et al. 2005; Irwin 1968; Moser

Table 1 Irwin screen—test parameters

Parameter	Description
	Behavioral domain
Ataxia	A measure of coordination during locomotion. Animals unable to maintain balance and walk on a rod are considered ataxic
Reactivity	Degree of responsiveness to external stimuli, including responses to a new external environment, touch, sound, or sudden movement
Catalepsy	A lack of response to external stimuli. Assessed as a failure to correct an externally imposed abnormal posture. Animals are placed with forepaws on an elevated wire; posture holding time >10 s indicates catalepsy
Passivity/irritability	Passivity is a reduction in or absence of struggle when loosely restrained, which is considered the opposite of irritability. Irritability may also be associated with signs of aggression such as tail lashing, vocalization, or biting
Spontaneous motor activity	A measure of locomotor activity, rearing, grooming, and social interaction with other animals. May be seen to both increase and decrease
Stereotypy	Animals display repetitive, apparently purposeless behaviors such as head bobbing, shaking, gnawing, or licking. Includes normal behaviors when these are manifested to an abnormal degree
Mortality	The number of dead animals at each dose level and observation time
	Neuromuscular domain
Exophthalmia	Protrusion of the eyeball assessed visually
Gait	Described as hypotonic or hypertonic. Hypotonic gait is caused by muscle weakness or paralysis and is manifested by splayed posture or a swaying/waddling walk. Hypertonic gait is caused by increased muscle tone or spasticity and is manifested by elevation of the hind limbs with limited movement and a shuffling walk or tiptoeing
Grasping reflex	Measured using the wire maneuver which assesses the animal's ability to grasp and retain grip on the horizontal wire
Grip strength	Measured as grip resistance using grip meter device
Straub tail	Elevation of the tail from a horizontal (normal) to vertical position; in extreme cases the tail is held horizontally over the back of the animal
Tremor	Tremors are involuntary movements caused by alternating contractions of opposing muscle groups. They range from slight to pronounced and differentiated into exertion tremors observed during movement and resting tremors
Convulsion	Convulsions are violent involuntary contractions of voluntary muscles. There are two major types of convulsions: clonic in which the muscles alternately contract and relax and tonic in which a sustained contraction, cramp-like, is seen Convulsions may be mixed (clonic followed by tonic)
	Autonomic domain
Diarrhea	Loose or liquid stool
Lacrimation	Watering of the eye. Chromodacryorrhea (red-colored tears) may also be visible
Piloerection	Ruffling of fur or appearance of fur standing on the end

(continued)

Table 1 (continued)

Parameter	Description
Ptosis	Partial or full eye closure produced by drooping of the upper eyelid. Distinguishable from sleep-related eye closure since ptosis is not easily reversible upon disturbance
Pupil size	Determined using a binocular microscope. Miosis is a constriction of the pupil size, whereas mydriasis is the opposite
Rectal temperature	Temperature is recorded with probe. Hypothermia ≤ 36 °C, hyperthermia ≥ 40 °C
Salivation	Ranging from dry mouth to wet around the rim of the mouth or drooling
Urination	Ranging from excessive to unusually low
	Sensorimotor domain
Corneal reflex	Eyeblink response elicited by touching the cornea
Righting reflex	The response to restore the body to its normal upright position. Impairment of the righting reflex is measured by assessing the ability of the animal to land squarely on all four extremities when released from a supine position
Pineal reflex	The response to retract or twitch the ear and/or shake the head when a bristle is inserted into the auditory meatus
Tail pinch	The response to the pressure applied to the tail. A normal animal will respond by vocalization or attempting to escape. A decreased or absent response indicates analgesia
Hot plate	The reaction time when an animal is placed on a hot plate. The latency to paw or hind-leg licking or jumping is recorded. An increased latency indicates analgesia

et al. 1988; Gad and Gad 2003; Tegeris and Balster 1994; Porsolt et al. 2002; Gauvin and Baird 2008).

2.2 Data Analysis and Interpretation

The effects seen in an Irwin screen may be grouped by neurological function into four domains (Table 1). Most parameters are evaluated by their presence or absence. Some domains are evaluated or rated on a four-point scale. These ratings are 0 = no change in behavior, 1 = minor change, 2 = intermediate change, and 3 = major change relative to the vehicle group. Parameters such as rectal temperature and pupil diameter are measured quantitatively. For example, chlorpromazine is a standard neuroleptic agent that induces stereotypic, cataleptic, and hypothermic effects (Moscardo et al. 2007) in three different domains, behavioral, neuromuscular, and sensorimotor. In contrast, amphetamine produces characteristic stimulant, psychotomimetic, and hyperthermic effects (Redfern et al. 2005) in the same three domains. Although grouping data in the various domains is useful, one should be mindful that many behavioral parameters engage more than one domain such that simple associations between behavioral effects and single clinical endpoints cannot

be made. For example, most sensory testing available in these batteries involves either the assessment of the motor response to various auditory, nociceptive, or sensorimotor stimuli or the assessment of simple reflexes such as pineal or grasping reflexes. Likewise, assessment of neuromuscular function and coordination employs a wide range of paradigms to evaluate gait and posture, muscle tone, grip strength, and righting ability based on proprioceptive responses.

Irwin assay results could potentially inform an investigator about where to focus future drug discovery efforts to avoid a possible liability. Using this approach overcomes the issue of placing too much emphasis on a single readout and provides a means to collect diverse data into a smaller categorical array (Baird et al. 1997). The downside, however, is that if inappropriate parameters are grouped together into a domain, there is the risk of diluting the analysis of the data within the domain thereby decreasing the probability of identifying true effects. Therefore, any established observational battery should be detailed, standardized, and easily repeatable.

2.3 Strengths and Shortcomings of Behavioral Approaches

The most obvious benefit of using behavioral batteries such as the Irwin test to assess potential adverse CNS effect is that it incorporates a wide variety of endpoints and therefore enables a broader characterization of potential effects. Observational assays are also noninvasive and do not require expensive equipment or time- and resource-intensive training as is required for more specific behavioral or electrophysiological assays. These tests provide flexibility and the parameters used in an observational battery can change in order to focus on specific behaviors and/or test-specific hypotheses. Due to the widespread use of these tests over many years, large data sets are available which demonstrate the reproducibility and validity of these methods. Specifically, an international collaboration sponsored by the International Program on Chemical Safety (IPCS 1997) assessed the general utility and reliability of behavioral observation procedures (Moser et al. 1997a, b). Using a standardized test protocol, eight laboratories from government, academia, and the pharmaceutical industry evaluated the effects of a number of positive and negative control compounds using a functional observational battery. All laboratories were able to discriminate between these two compound sets demonstrating that the observational battery provided a consistent and robust measure of CNS effects. In a more detailed analysis, the investigators then developed a reliability index for each behavioral measure and compared them to assess the accuracy of individual endpoints in detecting adverse events (Moser 2000). Some individual parameters such as tail pinch (nociception) or gait abnormalities were good indicators of CNS adverse events, whereas others, such as piloerection and urination, were not. This is consistent with the recommendation that behavioral observations in multiple endpoints may be more indicative of a potential CNS safety risk than an isolated effect on one parameter. An additional advantage is that, due to the diversity of parameters measured, it may be possible to identify potential

pharmacological mechanisms using Irwin/FOB data. For example, increased salivation, lacrimation, urination, and/or diarrhea would suggest muscarinic receptor agonism (Witkin 1990).

The main weakness of behavioral testing is that some endpoints do not translate directly to CNS adverse events in humans. For example, Straub tail, rearings, and piloerection do not have human equivalents. Many drugs that cause hypothermia in rodents do not alter temperature readings in humans. This is an issue in CNS safety assessment generally since, due to large differences in rodent and human behavior, complete concordance cannot be assumed. Since most behavioral endpoints are subjective, it is unsurprising that comparison of baseline measurements between laboratories shows significant variation and that, in spite of attempts to train observers to rate behaviors consistently, even within the same laboratory, different observers can interpret behaviors differently (Moser et al. 1997a, b). The scope for intra- and interlaboratory differences highlights the need for proficiency studies to confirm the ability of investigators to measure subtle and reliable behavioral endpoints. Another consideration is that behavioral changes may be due to a peripheral mechanism of action. A retrospective analysis of FOB data for 50 non-CNS-targeted compounds demonstrated that the most common outcomes were weight loss or reduced body weight gain and core body temperature changes (Redfern et al. 2005). If a compound has no CNS permeability, it is reasonable to speculate that changes in body weight and temperature are related to peripheral mechanisms of action.

Although behavioral observational tests are informative, they do not represent a complete assessment of drug effects on the CNS (Ross 2000). The Irwin or FOB tests do not assess the senses including taste, visual, olfactory, and auditory systems, as well as drug effects on significant risks such as seizure potential, sleep disruption, cognitive impairments, or abuse potential. The ICH S7A guideline stipulates the need for follow-up studies as warranted by outcomes of CNS core battery experiments or other preclinical studies. In addition, when possible, endpoints should be less subjective and quantifiable and have direct translation to eventual clinical endpoints. In agreement with this, behavioral endpoints together with electroencephalogram (EEG)-based techniques are described below as definitive assays to assess the potential for drug-induced seizures or sleep disruption. Given the current drive within the pharmaceutical industry to develop new, higher-throughput screens for adverse effects, we also discuss the potential use of in vitro brain slice electrophysiology in CNS risk assessment. As with all areas of preclinical safety assessment, the eventual aim is to design testing cascades that flag potential CNS risk using in vitro assays prior to testing in more resource-intensive in vivo assays. In the remainder of this chapter, we describe assays to evaluate the potential for three of the most significant CNS adverse events: seizure, sleep disruption/sedation, and cognition deficits which are not assessed directly using standard behavioral endpoints. This is not intended to give an exhaustive review of the information available but to highlight examples where more specific and/or quantitative models may add value to the traditional behavioral approaches used within CNS safety pharmacology.

3 Seizure Risk Assessment

Seizure is defined as the abnormal synchronous firing of large populations of neurons that may result in involuntary movements, including a clonic–tonic convulsion and/or altered state of consciousness. It is worth noting that there are convulsion-like motor behaviors that are not seizures as well as seizures that are not accompanied by convulsions or other motor behaviors (Kaplan 1996). Drugs that induce seizures are thought to act by directly or indirectly causing excessive inhibition of inhibitory neural circuits, such as those mediated by GABA neurotransmission, or by stimulating excitatory circuits, such as those mediated by glutamate neurotransmission. Most seizure-inducing drugs cause excessive neuronal excitation by acting directly in the brain (Jain 2001; Zaccara et al. 1990). However, it is also possible for non-blood–brain-barrier-penetrating drugs to trigger seizures by inducing hyperglycemia, hypoglycemia, hypotension, or other drastic perturbations of energy balance or metabolism, which in turn may result in abnormal neuronal activation (Delanty et al. 1998). Compounds associated with seizurogenic potential span a wide variety of pharmacological classes and therapy areas, including many not targeted at the CNS (Zaccara et al. 1990). In a recent review it was described that the largest therapy area represented within a group of 275 seizure-associated agents was CNS disorders (35 %). Agents associated with other therapy areas were also well represented, including infection (16 %), cardiovascular (14 %), respiratory (12 %), and metabolism (6 %) (Easter et al. 2009). Although marketed drugs can cause seizures in some patients, these seizures generally occur at doses/exposures that are significantly higher than the recommended therapeutic ranges. Such exposures may occur during drug overdose (Thundiyil et al. 2007), in patients with compromised blood–brain barrier function (Friedman 2011), or in situations where renal or liver function is reduced resulting in decreased drug metabolism (Marinella 1998). In addition, patients with a genetic predisposition to epilepsy may be more susceptible to drug-induced seizure at lower exposures than non-epileptic patients.

Drug-induced seizure is a potentially life-threatening adverse drug reaction that can consequently result in the failure of drugs to be licensed for use or withdrawn from the market. Surprisingly, there are no regulatory guidelines describing how drug-induced seizure should be addressed preclinically, while CNS safety pharmacology studies performed as part of regulatory submissions are only mandated to assess general behavioral effects as outlined in the ICH S7A guidelines (Guidance for industry: S7A safety pharmacology studies for human pharmaceuticals. US department of health and human services food and drug administration center for drug evaluation and research (CDER) center for biologics evaluation and research (CBER) 2001). However, due to the serious potential consequences of drug-induced seizures, a number of optional follow-up studies may be used to address this issue, and, depending on the therapy area and patient population being targeted, significant effort is expended in preclinical safety assessment to identify and mitigate seizure risk. Such studies are usually undertaken if there are risk flags that would indicate seizure potential during early discovery, such as (a) existing

Table 2 Behavioral scores of preconvulsive and convulsive behaviors

0 = no abnormal motor signs
1 = facial or mouth movements
2 = myoclonic jerk or abnormal head movements
2.5 = forelimb/hind-limb flexion and then extension
3 = forelimb clonus
4 = loss of righting reflex
5 = tonic–clonic convulsion

data suggesting that the compounds from that chemical or drug class are seizurogenic, (b) evidence or knowledge that the compound penetrates the blood–brain barrier, (c) pharmacological profile indicating that the compound interacts with molecular targets associated with seizure risk, and/or (d) any in vivo behavioral observation suggesting convulsions or other abnormal CNS effects (Easter et al. 2009).

3.1 Convulsion Observations in Irwin/FOB

Behaviors associated with convulsions in species such as rodents, dogs, and non-human primates share many characteristics with those seen in humans. In rodents, convulsions are often preceded by other behaviors such as stereotypies (head bobbing, scratching, excessive grooming), twitches, and a general loss of locomotion and exploratory behaviors (Racine 1972). Myoclonic jerks, wild running, and loss of the righting reflex may be observed immediately before the onset of convulsions. Convulsions can be classified as (a) clonic, which involves the rapid movement of the forelimbs and is often accompanied by facial twitching, or (b) tonic, which consists of the sustained contraction of muscle groups in the torso and extending toward the hind and forelimbs (Moser et al. 1995; Veliskova 2006). In the most extreme cases, convulsions may be mixed, by which clonic movements are followed by tonic contractions; on occasion clonic–tonic convulsions can be lethal. Table 2 shows a behavioral scale to score preconvulsive and convulsive behaviors based on the severity of motor manifestations, modified from Racine (1972).

As mentioned previously, there are behavioral manifestations that may be mistaken for a seizure-associated convulsion, and thus it is strongly recommended that observations of preconvulsive or convulsive behaviors be followed up with an EEG study to confirm the occurrence of electrographic seizure.

3.2 Pentylenetetrazol (PTZ) Pro-convulsant Assay

PTZ is a $GABA_A$ receptor antagonist that is widely used to study the susceptibility of drugs to alter convulsive threshold [reviewed in Loscher (2009)]. As a direct inhibitor of GABA systems in the brain, PTZ is itself a potent seizurogenic

compound. In contrast to assaying a drugs' ability to trigger seizures on its own, PTZ can be administered in combination with the test article to assess pro-convulsant potential. Pro-convulsant compounds are defined as those compounds that lower seizure threshold. In that context, information derived from the rodent PTZ assay may help in volunteer or patient selection during clinical trials by excluding individuals with predisposing factors for seizure such as epileptic patients. In addition, it is recognized that pro-convulsant compounds as identified with the PTZ assay, such as venlafaxine, fentanyl, or isoniazid, may also be convulsant when administered alone, and thus the PTZ assay can be used as an indirect preclinical measure of convulsant risk (Himmel 2008; Santos et al. 2002). However, not all convulsant compounds cause pro-convulsant effects; the antidepressant bupropion which has a well-established seizurogenic potential in the clinic does not alter seizure threshold in the PTZ assay (Tutka et al. 2004). The PTZ assay begins with the administration of the test article to rats or mice, followed by the timed intravenous infusion of PTZ. Latency to convulsion onset is proportional to the pro-convulsant potential of the test article.

3.3 Electroencephalography for Seizure Detection

Because the use of the electroencephalogram (EEG) for seizure detection is discussed in detail in a separate chapter, we will limit our description here to a few basic concepts to provide some context for comparison with other seizure detection assays. Given that a seizure is defined as abnormal synchronous neuronal activity, it can be detected by surveying the brains' electrical activity. The EEG is the definitive seizure detection assay both preclinically and clinically. EEGs can help determine, beyond behavioral ambiguities, if a given drug causes seizure and at what doses or exposures. A detailed description of seizure detection with mouse EEG is described by Fonck and collaborators (2005). A seizure recorded from the cortex of any mammalian species typically manifests in the EEG trace as repeated spike waves (Abou-Khahil and Misulis 2006). These spikes are thought to represent the summated synchronous firing of pyramidal neurons that span the cerebral cortex of all mammals. Regardless of where or how a seizure is initiated, drug induced, physical trauma, or epilepsy, once it generalizes to include the cerebral cortex, the spike-wave morphology is easily recognizable. There are other EEG waveforms such as paroxysmal spikes, fast ripples, or postictal depression that can serve to confirm the ictal nature of observed EEG abnormalities. Abundant literature supports a high degree of translation across mammalian species, from rodents to humans, when assessing the seizurogenic potential of drugs using EEG. To our knowledge, there are no published reports of drugs that have triggered seizures in humans that have not done so in rodents when tested. For certain drug classes, it is possible to detect drug-induced changes in EEG frequencies which can serve as translatable safety biomarkers (Fig. 2, unpublished results). Because changes in EEG frequencies are not decipherable with the naked eye, it is necessary to perform spectral analysis of raw EEG data by implementing a fast Fourier transform (FFT).

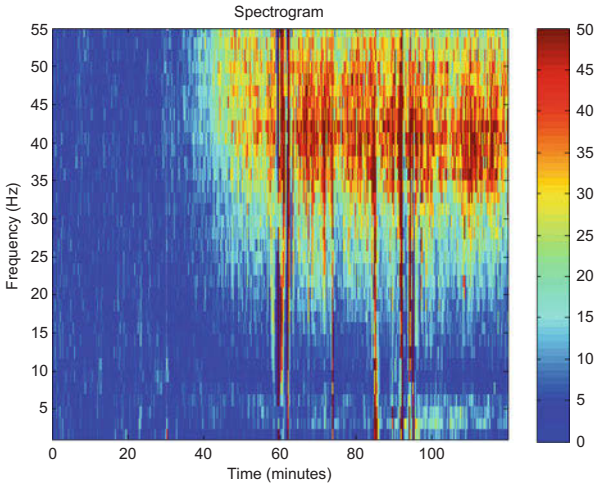

Fig. 2 *EEG spectral changes as a seizure safety biomarker*. Heat map shows spectral changes from EEG recordings in mice ($n = 5$) dosed with memantine 25 mg/kg i.p. Baseline recordings lasting 30 min are delineated by *white dotted line*. Frequency changes are calculated with a fast Fourier transform. Memantine causes a significant increase in gamma (>30 Hz) and beta (13–30 Hz) frequency density at sub-seizure threshold doses (≤25 mg/kg i.p.), as shown by *dark red coloration* on the heat map. Data are unpublished

Our group recently developed an automated blood sampling and telemetry (ABST) system that combines EEG recordings with other physiological parameters such as blood pressure or core temperature (Kamendi et al. 2010). The ABST system is advantageous to determine the sequence of adverse effects and perhaps the causality of these, when, for instance, the test article demonstrates both CNS and cardiovascular effects. Van der Linde and collaborators also developed a model combining EEG and ECG recordings in fentanyl/etomidate-anesthetized beagle dogs (Van der Linde et al. 2011).

3.4 In Vitro Electrophysiology and Seizure

Brain slices maintain some of the neuronal circuitry and therefore electrophysiological properties as the intact brain (Lynch and Schubert 1980) and are a useful tool to investigate how molecules can modulate brain function, either to drive efficacy (Cho et al. 2007) or to induce CNS adverse events (Fountain et al. 1992). Brain slice electrophysiology has been used extensively in the field of epilepsy research both to investigate the mechanisms underlying seizure induction and propagation and also in antiepileptic drug discovery to assess the efficacy of preclinical candidate drugs. Typically, seizure-like activity is induced by application of drugs known to induce seizures in vivo, and the ability of potential anticonvulsants to inhibit such seizure activity is assessed (Ashton et al. 1988;

Sagratella 1998). Less commonly, these techniques have been used to investigate the seizurogenic potential of preclinical drug candidates. To date, most work in this area has focused on the hippocampal brain slice, largely due to a defined cytoarchitecture that makes it amenable to electrophysiological recording (Dingledine et al. 1980). The hippocampus is also strongly linked to partial seizures, including temporal lobe epilepsy (Schwartzkroin 1994). Using single-microelectrode field-potential recording of evoked synaptic activity from hippocampal brain slices, it has been shown that a number of standard seizurogenic compounds, including 4-AP (Southan and Owen 1997), picrotoxin (Hablitz 1984), and PTZ (Leweke et al. 1990; Rostampour et al. 2002), cause strong excitatory effects resulting in increased firing in neuronal cell populations. In addition, similar effects have been seen with a number of drugs associated with seizure clinically including antibiotics (Schmuck et al. 1998), antipsychotics (Oliver et al. 1982), X-ray contrast agents (Frigeni et al. 2002), and antidepressants (Luchins et al. 1984). Similar results have been reported using multi-slice recording systems (Easter et al. 2007) which allow increased experimental throughput, although this is still low relative to cell-based in vitro assays. An additional development is the use of multielectrode arrays which consist of a grid of several recording sites, which allow the simultaneous recording of electrical activity across a brain slice (Gonzalez-Sulser et al. 2011). Multielectrode arrays typically have 64 recording sites, and multiple arrays can be combined to increase throughput further, up to 200 compounds per day according to a recent estimate (Johnstone et al. 2010). This can give more mechanistic information regarding the focus and spread of electrical activity. The above techniques may also be adapted to recording from cell populations, either primary cell cultures, stem-cell-derived neuronal cultures (Illes et al. 2007), or mixed organotypic cultures (Berdichevsky et al. 2009) that retain some of the synaptic connections found in intact brain slices. Seizurogenic compounds have been shown to induce synchronous firing in organotypic cell cultures using multielectrode array recording (Routbort et al. 1999). In addition to increasing experimental throughput, a further advantage is that cells can be maintained in long-term culture whereby one preparation can be used multiple days facilitating investigation of the effects of chronic drug exposure. Although most of the work in this area has focused on hippocampal brain slices, compounds may elicit seizure in a number of different brain regions; e.g., generalized seizures are thought to result from changes in the electrical activity of thalamocortical circuits which lead to simultaneous disruption of normal brain activity in other brain areas (Gloor and Fariello 1988). In agreement with this, seizure-like activity has also been recorded from neocortical (Voss and Sleigh 2010) and thalamocortical (Gibbs et al. 2002) brain slices. For this reason, there may be utility in investigating the use of non-hippocampal brain slice preparations; the most appropriate preparation is likely to be dependent on the exact mechanism of drug-induced seizure.

As described above, the throughput and resource requirements of these electrode-based slice techniques make them unsuitable for high-throughput screening in early drug discovery. In cases where there is evidence that drug-induced seizure is due to modulation of a specific molecular target, it may be possible to use

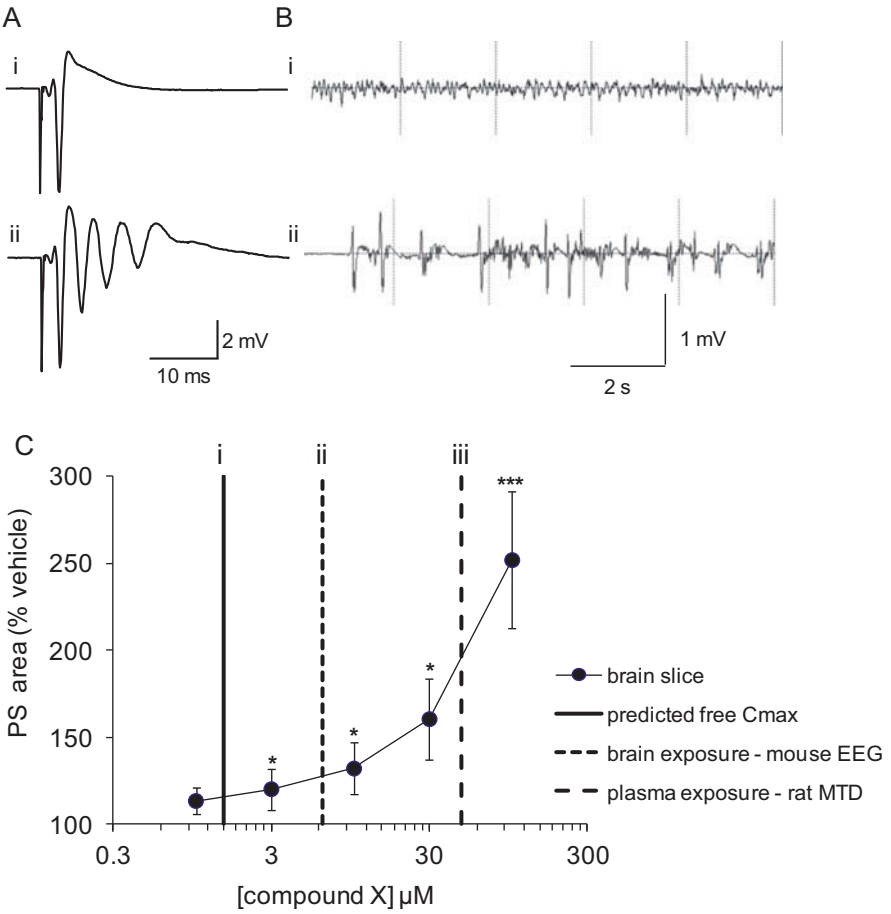

Fig. 3 *Use of electrophysiological assays in preclinical seizure risk assessment*. Compound X was a small molecule from a non-CNS-targeted drug discovery project. During a rodent maximum tolerated dose (MTD) toxicology study, this compound caused convulsions therefore triggering testing in follow-up seizure assays. (**a**) *Mouse hippocampal brain slice*. CA1 population spikes (PS) were recorded as described previously (Easter et al. 2007). Representative PS recording from one slice in the presence of (i) vehicle and (ii) 100 µM compound X. Note the appearance of additional population spikes due to a direct excitatory effect on synaptic transmission. (**b**) *Mouse EEG*. Example trace from a mouse implanted with cortical electrodes following administration of (i) vehicle or (ii) 400 mg/kg compound X. Spike-wave activity was contemporaneous with forelimb extension, Straub tail, shaking, and convulsion. (**c**) Integrated data summary of compound X effects in preclinical seizure assays. 1–100 µM compound X evoked a clear concentration-dependent increase in PS area (*filled circle*; $*P < 0.05$, $***P < 0.001$). *Line* (i) indicates the estimated efficacious human free C_{max} plasma exposure, and compound X was inactive in both brain slice and EEG at this exposure. Also shown are exposures measured during in vivo studies: (ii) *dotted line* indicating brain exposure associated with seizure activity in the EEG study and (iii) *dashed line* indicating the free plasma exposure associated with convulsion in the MTD study. Note that the in vivo exposure values overlap with the concentrations of the compound which evoked significant effects in the in vitro brain slice assay. Following demonstration of in vitro–in vivo concordance, the brain slice assay was used to select additional candidate compounds. Data shown are unpublished

higher-throughput electrophysiology or other in vitro techniques in preclinical safety screening. An example of this would be in the screening of beta-lactam antibiotics where activity at recombinantly expressed $GABA_A$ receptors has been shown to correlate with brain slice electrophysiology and in vivo data (Sugimoto et al. 2003). Where in vitro–in vivo concordance is established, it is then possible to screen compounds using higher-throughput in vitro assays which can assess compound activity at a single target (Fig. 3). As more knowledge surrounding the pharmacological mechanisms underlying drug-induced seizure becomes available, it is likely that electrophysiological screening of recombinantly expressed targets will become more common in this area.

4 Drug-Induced Sleep Disruption

Drug-induced sleep disruption has been well documented since the introduction of barbiturates as hypnotics in the 1970s (Stotsky et al. 1971). From a drug-safety perspective, the main concern with drug-induced sleep disruption is the consequent daytime drowsiness that follows, which in turn may lead to increased risk of work- or driving-related accidents. Long-term chronic sleep disruption, drug induced or otherwise, has been linked to obesity (Cappuccio et al. 2008), cardiovascular disease (Cappuccio et al. 2011), and depression (Nakata 2011). Several drug classes are associated with sleep perturbation including antidepressants, antipsychotics, corticosteroids, and antihistamines. Drugs can interfere with sleep by impacting some or all of the sleep stages; in general, drugs that suppress the slow-wave stage (SWS), also called restorative sleep, are regarded as the most detrimental (Novak and Shapiro 1997). Drugs that have central stimulant effects such as the appetite suppressant pseudoephedrine or the corticosteroid prednisolone can reduce all stages of sleep including SWS (Berkowitz et al. 1989). Opioid analgesics and antidepressants can impact sleep by primarily suppressing the rapid eye movement (REM) stage of sleep (Novak and Shapiro 1997). The question of whether a net reduction in REM sleep alone can negatively impact overall health remains a subject for debate. Some antidepressants like amitriptyline and the beta-blocker propranolol have been associated with an increase in parasomnias, which are sleep disturbances commonly described as sleepwalking or sleep terrors (Thompson and Pierce 1999). Drug-induced sedation is considered an adverse effect for reasons akin to those for sleep disruption. In this context, it is worth noting that hypnotics, which are drugs that induce sleep, often have overlapping effects with sedatives, which are drugs intended to reduce irritability or excitement.

In general, drug-induced sleep disruption is not regarded as a serious safety risk when considering progression to a phase I clinical trial; however, for certain indications and patient populations, drug-induced sleep disruption may be an unacceptable adverse effect. In this context, it is useful to consider the benefit of studying drug-induced effects on sleep in preclinical models. Given that all mammalian species have measurable sleep/wake cycles, there is the expectation that rodent or non-rodent species used in standard safety assessment studies can serve as

animal models to study drug-induced sleep disruption in human. There is a significant body of literature that supports the notion that most, if not all, drugs that perturb sleep in humans do so in other mammalian species. It is also important to consider differences in sleep architecture across species when deciding which aspects of preclinical sleep assessment might translate into the clinic. For example, humans typically have two bouts of SWS in a normal night's sleep, while rodents have multiple sleep–wake transitions, with several dozen discrete periods of SWS distributed in a 24-h cycle. In other words, sleep architecture in rodents is significantly more fragmented compared to that of humans. Nonetheless, rodents, dogs, nonhuman primates, and people share the main vigilance states: (a) an awake phase associated with a conscious state in humans, (b) a REM stage which is a sleep/wake transition period during which humans report having dreams, and (c) a non-REM (NREM) period that includes SWS which is thought to be the most important contributor to achieving a state of restfulness.

4.1 Behavioral Approaches to Assessing Sleep Disruption

Behavioral assays to study the effect of drugs on sleep consist of observing and/or measuring activity patterns of animals. In this sense, behavioral assays are actually measuring drug effects on circadian rhythm rather than on sleep. Circadian rhythm refers to any of many biological processes, including behaviors, which occur in a 24-h cycle and are controlled by an endogenous but entrainable pattern generator residing in the suprachiasmatic nucleus of the hypothalamus. It is well established that disruptions of the circadian rhythm often impact the quality and quantity of sleep (Franken and Dijk 2009). The most popular behavioral assays to study changes in circadian rhythm are the locomotor activity assay and the wheel-running assay, both of which are typically performed with rodents. The locomotor activity assay involves measuring horizontal movement of mice or rats in their home cage over many hours or days. Most locomotion detection systems are based on the interruption of infrared beams detected by photocells on the outside of a transparent cage. It is assumed that animals are awake and active during periods of high beam-break counts. The wheel-running assay is another method by which circadian locomotor activity can be tracked. Compared to other behavioral assays such as the Irwin test, the locomotor activity and wheel-running assays have a number of advantages: they are relatively easy to implement, experimenters need little or no training, and data are objective and quantitative allowing for direct statistics-based comparisons across compounds. Drugs known to prevent or reduce sleep in humans such as sibutramine, modafinil, and ephedrine trigger a strong signal in the locomotor activity or wheel-running assays (Meng et al. 1999; Kim et al. 2005). Likewise, drugs that promote sleep or cause sedation in humans such as diazepam, zolpidem, and diphenhydramine will readily cause a decrease in locomotor activity in rats and mice (Kalivas 1982; Vlainic and Pericic 2009).

4.2 Sleep EEG

Behavioral approaches to evaluate drug-induced sleep disruption are limited by their inability to distinguish between lack of motor activity and sleep. In addition, drug-induced effects on the cardiovascular system and the respiratory system or any effect resulting in a general decrease in metabolism may cause a reduction in behavioral activity which could be misinterpreted as a direct CNS effect. As is the case with the evaluation of drug-induced seizure, EEG is the most definitive assay for both preclinical and clinical assessment of drug-induced sleep disruption. Furthermore, the terminology used to describe specific stages of sleep is based on changes in the wave form and frequencies of cortical EEGs. In humans as well as in rodents, dogs, and nonhuman primates, wakefulness manifests in the EEG as oscillations in alpha (8–13 Hz), beta (14–30 Hz), or gamma (>30 Hz) frequency bands. Depending on the degree of alertness or activity, EEG frequencies shift between alpha and higher frequency bands. Sleep in all mammalian species is composed of two main stages: NREM sleep which fluctuates between delta (0.5–3 Hz), theta (4–8 Hz), and alpha frequencies and REM sleep which occurs in the theta–alpha frequency range (6–9 Hz). NREM sleep is divided into N1 which occurs during sleep onset, N2 which is characterized by sleep spindles (quick burst of low-amplitude spikes) and K-complexes (large-amplitude peaks), and N3 which is also called SWS or deep sleep and is dominated by delta frequency. The resulting spectrogram is typically scored manually by designating vigilance states based on the dominant EEG frequencies over the time course of the experiment as identified by FFT analysis (Fig. 4, Fonck et al. 2005). Although there are some systems that provide automated sleep scoring, most experts agree that manual scoring is still more accurate, in particular when video feed is available to confirm vigilance states.

There are a number of commercially available systems to perform mouse or rat EEG sleep studies. EEG-recording electrodes are typically implanted bilaterally in

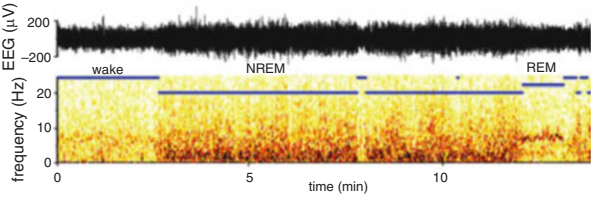

Fig. 4 *Sleep EEG recording and analysis*. Example of an EEG trace (*top* of figure in black) with its corresponding spectrogram from a C57BL6 mouse during baseline recording. Power density changes in the spectrogram are represented by the color intensity of individual pixels: dark coloration means high-power density. The vigilance state of the mouse was determined with a computer algorithm that calculated power spectrum changes over time. The *blue line* in the *upper portion* of the spectrogram represents the output of the algorithm, which is confirmed by visual inspection of the raw EEG trace and video feed of the mouse. This 15 min segment shows periods of wake and NREM and REM sleep (Fonck et al. 2005). Permission granted by Journal of Neuroscience

the parietal, motor, or somatosensory cortices. The signal required for sleep/wake EEG assessment is robust across several regions of the cortex. In addition, it is recommended that an electromyogram lead be placed on the nuchal (neck) muscle to detect changes in muscle tone which serve to distinguish between awake and sleep states, in particular REM. Prior to drug administration, a baseline recording session lasting at least 24 h, but preferably longer, is used to establish sleep–wake baseline of a specific cohort of animals. In studies to determine the acute effect of a drug on sleep, the postdrug recording period should last another 24 h because of a potential rebound effect. Rebound effect refers to an increase in sleep time following a period of excessive wakefulness. Similarly, EEG recordings for a chronic drug regimen should last 24 h past the last dose of compound. In addition to measuring sleep and wake time, it is important to quantify the number of sleep–wake transitions as a measure of drug-induced sleep fragmentation. Spectral analysis also serves to quantify the amount of delta (0.5–3 Hz) activity that occurs during NREM sleep. NREM delta power is a measure of the sleep homeostatic drive, which is thought to represent how tired the animal or person is. In addition to running a vehicle control group, it is useful to have a positive control group tested with compounds such as modafinil, which is a powerful pro-wakefulness drug, or alternatively, and depending on the expected effect of the test compound, with zolpidem which is a commonly used sleep aid.

4.3 In Vitro Sleep and/or Sedation Assessment

As for other CNS adverse events, there has been limited use of in vitro techniques to investigate drug-induced sleep disruption. This is most likely due to the complex network activity required to generate the changes in neuronal firing pattern that ultimately result in EEG frequency changes. However, brain slice electrophysiology represents one area which may be promising in terms of developing in vitro screens to assess this adverse event.

As discussed previously, there has been considerable clinical research to investigate the EEG patterns associated with sleep disruption, and in vivo studies demonstrate that these changes are translated in preclinical species. Less is known about how these EEG observations correlate with electrical activity in in vitro brain slices. Since clinical EEG recordings represent the total synaptic activity of the upper layers of the cerebral cortex, much research in this area has focused on thalamocortical brain slices, although hippocampal slices have also been used. It has long been known that rhythmic network-driven oscillatory activity can be recorded from in vitro brain slices and the frequency of this activity is similar to the EEG frequencies associated with sleep. For example, theta rhythm activity has been recorded from cortical (Lukatch and MacIver 1997) and hippocampal (Konopacki et al. 1987) brain slices, and similar activity has also been recorded from organotypic slice cultures (Fischer et al. 2002). Furthermore, anesthetic agents depress action potential firing and oscillatory network activity in neocortical brain slices (Lukatch et al. 2005) and organotypic cultures (Antkowiak and Helfrich-

Forster 1998). Similar effects were seen with benzodiazepines (Drexler et al. 2010) and barbiturates (Lukatch and MacIver 1996) which are used clinically to induce sedation. To date, brain slice assays have not been utilized in the assessment of potential sedative effects, and this is unlikely to be used as a routine screen in preclinical safety assessment. However, in cases such as those discussed above, where in vitro correlates of in vivo effects are seen, brain slice studies could be used to gain mechanistic understanding of a sedative effect or to enable comparison of compounds from different chemical series or classes. Furthermore, if mechanistic studies revealed a potential mechanism of drug-induced sleep disruption (e.g., identified the involvement of a specific receptor), it would be possible to develop much higher-throughput cell-based assays to screen out this activity during chemical design.

5 Drug-Induced Cognitive Impairment

Drug-induced cognitive impairment refers to a number of symptoms by which patients report being confused, unable to recall memories, having difficulty performing computational tasks, or experiencing a loss of verbal fluency following drug treatment. From a mechanistic point of view, drug-induced cognitive effects can be separated into those that disrupt cognitive function following acute drug exposure and those effects that emerge following a chronic regimen. In the acute effects category, drugs such as benzodiazepines to treat anxiety or opioids used as analgesics can cause confusion or even delirium following a single dose (Tune and Bylsma 1991). Given the importance of the balance between neuronal excitation and inhibition, it is not surprising that drugs such as diazepam, which cause excessive inhibition by stimulating the GABAergic system, would disrupt cognitive function. With regard to chronic effects of drugs on cognition, drugs such as tricyclic antidepressants, for example, amitriptyline (Timoshanko et al. 2001), or antiepileptics such as phenytoin (Jokeit et al. 2005) may cause cognition deficits that become progressively more apparent following several doses. An area that has garnered significant interest recently stems from the observation that a large proportion of chemotherapy patients, up to 30 % in patients treated for breast cancer (Tannock et al. 2004), experience cognitive deficits that can persist several years beyond the time therapy ended. This condition is referred to as "chemo brain." Mounting evidence suggests that chemo brain involves damage to or loss of neuronal populations important for cognitive function (Mustafa et al. 2008).

As the study of mechanisms responsible for learning and memory in people and animals has been at the center of much of neuroscience research, the number of techniques and tools available to explore agents that impact these brain functions is overwhelming. In practice, however, evaluation of drugs that may impair cognitive function is rare relative to efforts to identify drugs that enhance cognition. Many cognitive function assays are based on the ability of rodents to learn and display complex behaviors. A challenging aspect of evaluating drug-induced effects on learning and memory arises from the inability of many assays to discriminate

among several discrete processes by which animals express learned behaviors: sensory input, memory formation and consolidation, memory recall, and ability to perform a motor task. For instance, a candidate drug that causes a loss of visual acuity may impair a rat's ability to map and find the standing platform in the Morris water maze, which ultimately could be mistaken for a sign of drug-induced impairment of working memory. More mechanistic approaches to assessing drug-induced cognitive impairment involve the use of in vivo or in vitro electrophysiological and imaging techniques that probe specific brain regions and neuronal circuits. For example, event-related potential (ERP) techniques, which are based on detecting EEG-measured responses to external stimuli, complement behavioral studies by providing a quantitative approach to track neuronal network activity during the acquisition and processing of information in the brain. In vitro brain slice electrophysiology may be used to investigate the sustained changes in synaptic transmission, often referred to as synaptic plasticity, which may underlie cognitive function. More so than in the assessment of other types of drug-induced CNS adverse effects such as seizure or sleep disruption, drug-induced cognitive impairment calls for a comprehensive approach by which behavioral assessment should be combined with electrophysiological and imaging techniques, while neuro-histopathology could help identify damaged or lost neuronal populations important for cognitive function.

5.1 Behavioral Assessment of Drug-Induced Cognitive Impairment

The Irwin or FOB tests do not evaluate the effects of new chemical entities on learned behaviors, and thus there is a need to deploy specialized behavioral assays to assess the potential for drug-induced cognitive impairment in preclinical species. The simplest models of memory formation describe a two-step process by which newly acquired sensory information enters short-term memory, and it then undergoes a process referred to as memory consolidation and eventually becomes long-term memory. In this scheme, short-term memory is retained for a few seconds, while long-term memory is retrievable over extended periods of time, sometimes through a lifetime. In contrast to humans, whereby a person may be asked to describe a memory (declarative memory), assessment of memory function is more challenging in preclinical species. Nonetheless, researchers in the field believe that approaches such as classical conditioning, passive avoidance, or the Morris water maze represent close approximations to evaluating the acquisition and retention of a learned behavior (Morris et al. 1982; van der Poel 1967).

Classical conditioning is the acquisition of a behavioral response to a new stimulus by association with a previous stimulus. Contextual fear conditioning is a form of classical conditioning used to evaluate the learned response to an environment where the animal previously experienced an aversive stimulus. For example, rats "freeze" when placed in a chamber where they have previously been subjected to an electric shock. This freezing response is either absent or reduced in

an animal with a lesion in the hippocampus or exposed to a drug that interferes with memory consolidation (Crawley 2000; Barak and Weiner 2011). Similarly, in the passive avoidance test, the animal is trained to withhold a response to avoid punishment (Barak and Weiner 2011). Passive avoidance learning involves not only declarative memory, which is hippocampus dependent, but also amygdala-dependent emotional memory: "fear of the shock box" (Grossman et al. 1975). Zaleplon, triazolam, and chlordiazepoxide are compounds known to lessen the step-through latency in passive avoidance (Noguchi et al. 2002). The Morris water maze is a popular behavioral assay to study spatial learning and memory (Morris et al. 1982). Rats or mice are placed in a circular pool with a hidden submerged platform that provides relief from swimming and the aversion rodents have for water. Animals need to properly map the platform location in order to gain access to an exit ramp. Like most learning and memory assays, the Morris water maze is also dependent on a functional hippocampus as demonstrated in lesion experiments (Eichenbaum 2000). An animal's use of specific events, cues, and reference points to find the platform are all considered types of declarative memory. Morris water maze studies have revealed learning deficits in animals exposed to (+)-methamphetamine (Vorhees 1996), to the NMDA antagonist AP-5 (Ferretti et al. 2007), and to several other drugs.

5.2 Evoked and Event-Related Potentials

When stimulated by a discrete event, such as a flash of light or a brief sound, the nervous system translates the information by synchronously firing action potentials in specific groups of neurons, starting, for instance, with cone cells in the retina. As information propagates through sensory pathways and into information-processing centers in the brain, discrete neuronal populations are activated in sequence, generating evoked electric potentials (EP) that can be detected with in vivo electrophysiological techniques. The timing and amplitude of these EPs, and how they can be disrupted by disease, injury, or drugs, form the basis for using in vivo electrophysiology for the study of drug-induced cognitive impairment. For example, a loss of neurons in the auditory pathway, including the auditory cortex, would typically manifest itself as a decrease in the amplitude (measured in μV) in the peaks of the EP, whereas a loss in myelination or other insults that slow nerve conduction would result in the delayed appearance and disappearance (measured in ms) of relevant peaks. When studying stimulus-induced responses in the brain, in contrast to peripheral ones, EPs are referred to as event-related potentials (ERPs).

One important feature of ERP paradigms for the study of drug-induced cognitive impairment is their ability to distinguish between responses that reflect the physical attributes of a stimulus and those that represent the meaning or symbolism a person or animal assigns to the stimulus. The P300 potential (P designates a positive inflection in voltage, and 300 is the response latency in milliseconds from stimulus presentation) discovered by Chapman and Bragdon (Chapman and Bragdon 1964) varies in amplitude depending on whether a correct combination of numbers

resulting in a meaningful association is presented to a subject, whereas the intensity of flashing lights shown to the same person instead modulates the amplitude of other ERP peaks such as N200 (negative voltage inflection ≈ 200 ms poststimulus presentation). ERPs can be elicited via auditory, visual, somatosensory, or electrical stimulation. Selection of a particular ERP protocol may depend on how important a sensory modality is for a given species, for instance, rodents are more sensitive to auditory stimuli compared to visual ones. Although there are shifts in timing and amplitude, rodents share many similarities with humans in the various components of the ERP (Umbricht et al. 2004). In mice the P1, P2, P3, and N1 peaks are equivalent in stimulus and pharmacological response properties to human peaks P50, P200, P300, and N100, respectively, when using auditory ERPs, described by Connolly and collaborators (2004). In an effort to establish an electrophysiological model of chemotherapy-induced cognitive impairment, Gandal and colleagues (2008) studied the effect of chronic regimens of methotrexate or 5-fluorouracil in mice using an auditory ERP paradigm (Gandal et al. 2008). Chemotherapy-treated mice showed a significant impairment in sensory gating as evidenced by an increased response ratio between P1 and N1 amplitudes following presentation of auditory stimuli. Compared to behavioral assays, ERPs have the additional advantage of detecting stimulus-elicited changes in neuronal processing in the absence of a behavioral response.

5.3 In Vitro Electrophysiology and Cognition

The hippocampus is known to be critically important in memory formation (Squire 1992). In agreement with this, hippocampal damage or lesions have been shown to disrupt cognitive function in both preclinical species and humans (Scoville and Milner 1957). For this reason, the hippocampal brain slice has been used widely to investigate cognitive function. It has long been postulated that long-lasting changes in the strength of synaptic connections between neuronal populations may underlie this effect; this is usually referred to as synaptic plasticity and may result in a potentiation or depression of synaptic function. In terms of cognitive function, long-term potentiation (LTP) and depression (LTD) are the most critical (Malenka and Bear 2004; Lynch 2004), with LTP being the most investigated.

LTP was first reported in the perforant pathway of rabbit hippocampal slices (Bliss and Lomo 1973) and has since been described in multiple preclinical species both in vitro and in vivo. Memory formation in rodents, e.g., during exploratory behaviors, has been shown to be associated with theta rhythm neuronal firing (Buzsaki et al. 1989), and similar stimulation patterns have been shown to induce LTP in vitro brain slices. Therefore, it appears that some of the circuitry involved in memory formation in vivo is retained in vitro suggesting that brain slice electrophysiology may be a useful tool to investigate drug-induced cognitive impairment. In agreement with this, a number of compounds known to cause deficits in cognition in vivo have been shown to decrease the magnitude of LTP, such as benzodiazepines (del Cerro et al. 1992), muscarinic antagonists (Hirotsu

et al. 1989), glutamate receptor antagonists (Collingridge et al. 1983; Balschun et al. 1999), and dopaminergic antagonists (Kerr and Wickens 2001). All of the above experiments used traditional single-microelectrode field-potential recording, and more recently similar results have been obtained using multi-slice recording systems (Kroker et al. 2011). LTP has also been recorded from hippocampal slice cultures (Collin et al. 1997), which, unlike acute brain slices, can be maintained for several weeks enabling chronic exposure to compounds. There have been very few reports that LTP can be recorded from cultured primary neuronal cultures (Salter 2001) or stem-cell-derived neuronal cultures; this is presumably due to the lack of complex synaptic connections in these cultures.

Although brain slice electrophysiology has been used extensively in basic research to investigate the synaptic events underlying memory formation, it has not been widely used in preclinical safety assessment in the pharmaceutical industry. However, it has been used to assess the potential adverse effects of industrial or agricultural chemicals on cognitive function (Altmann et al. 2002; White et al. 2007), and it has been suggested that hippocampal brain slice recording is of potential value in the preclinical assessment of drug-induced cognition deficits (Fountain et al. 1992; Wakefield et al. 2002).

6 Concluding Remarks

The need to use a comprehensive approach combining various behavioral and electrophysiological methodologies to evaluate drug-induced effects is not surprising given the complexity of the CNS. Drug-induced CNS adverse effects are rarely traceable to the direct interaction between a compound and a single receptor or enzyme because the information-processing role the nervous system plays is contained in a vast and complex neural network. Behavioral observations remain an effective way to evaluate CNS function, but as described above, more direct measures of network activity using in vitro and/or in vivo electrophysiology provide more objective and quantitative endpoints, a deeper understanding of the neuronal mechanisms involved, better translation to clinical methodologies, and improved prediction of clinical outcomes. As data derived from these mechanism-based assays accumulate, the hope is that we develop a better understanding of the chemical structure–activity relationships that are ultimately responsible for CNS adverse effects.

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Preclinical Abuse Potential Assessment

Mary Jeanne Kallman

Contents

Abstract

Although laboratories have been conducting scientific evaluations of the abused drugs for many years, preclinical evaluations of the abuse potential of new drugs have been an integral component of new drug applications more recently. The development of a unified testing approach is crucial prior to initiating individual studies to address abuse potential. The core preclinical studies that will be required include a dependence/withdrawal study, an assessment of the discriminative cue produced by the new drug, and an assessment of whether the drug will

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be self-administered. This discussion is focused on the requirements for drug scheduling recommendations from the FDA and how to conduct the evaluations that will be used to make those recommendations and how to select parameter details such as preclinical species, test doses, test conditions, route of drug administration, comparator compounds, and behavioral test designs recommended.

Keywords
Abuse liability • Central nervous system • Conditioned place preference • Controlled Substance Staff • Discriminative stimulus • Drug dependence • Drug development • Intracranial self-stimulation • Self-administration

1 A History of Abuse Liability Testing

Abuse liability as a topic for modern scientific exploration and model development goes back to the 1960s. The focus of the work in this area was on the understanding of drugs that were known to be abused by humans by building a preclinical database on these known compounds. The early researchers in the area developed animal models for characterizing compounds with known abuse and impacting policy on the availability of clinically scheduled and unscheduled compounds. Research efforts were primarily directed toward understanding robust drug effects in abuse models with little emphasis on compounds in the Schedule IV category or compounds with minimal effects. Excellent reviews of the preclinical models are available and the characterization of drugs with human abuse should be consulted by those who need in-depth understanding of this earlier literature (Balster and Bigelow 2003; Brady 1991; Collins et al. 1984). From the human data perspective, these early investigators were eager to better understand the drivers for human abuse as a psychiatric phenomenon and to identify pharmacological tools for treating abuse. The primary emphasis of this chapter will be the reverse of this perspective where one is interested in using often the same techniques to predict abuse or identify the potential for abuse of new novel compounds entering the clinical market. Although drug development teams within the pharmaceutical industry are interested in some compounds with scheduling potential as Schedule 2, the majority of compounds in development are steered away from this level of scheduling. The goal of applying the data from these studies to drug scheduling for novel compounds is widely different than studies focused on characterizing compounds that are already known as abused compounds.

2 Reasons for Industry Interest in Abuse Potential

In the last 15 years, there has been a renewed interest by the pharmaceutical industry for the conduct of preclinical abuse liability assessments. The timeline of regulatory events from the Food and Drug Administration (FDA) and European Medicines Agency (EMA) has motivated the increased industry interests (see Fig. 1 for an illustration of the relationship between significant events affecting the

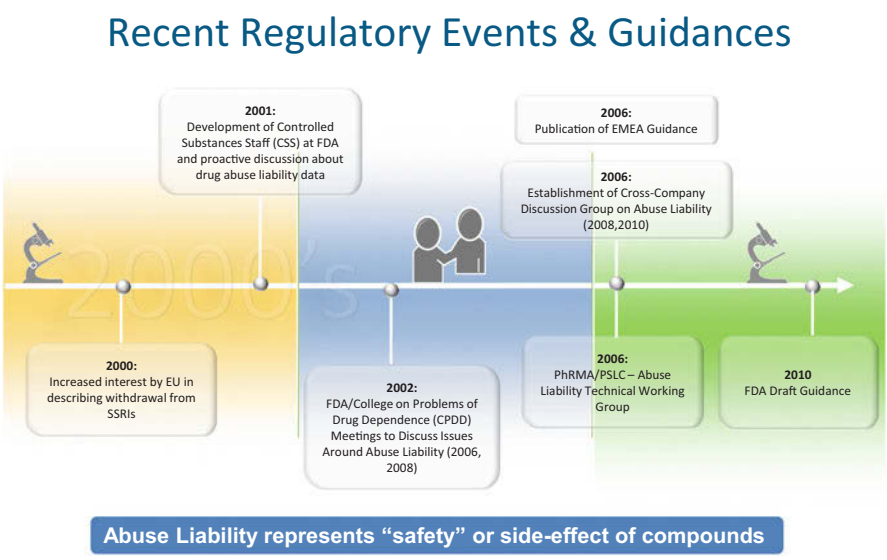

Fig. 1 Timeline for recent events impacting current regulatory requirements for abuse potential assessment

regulatory arena). The chain of events, as depicted in Fig. 1, was initiated by EMA concerns in 2000 over the withdrawal effects observed from the clinical use of selective serotonin reuptake inhibitors (SSRIs) that were not adequately described in preclinical studies. Although antidepressants are not scheduled compounds, issues related to tapering or discontinuing new antidepressant medications were not well understood. In 2001, soon after the issues with the SSRIs developed, the FDA formed the Controlled Substance Staff (CSS) to be advisory consultants for new drug applications (NDAs) at the FDA and advisory as well to the Drug Enforcement Administration (DEA) for scheduling decisions. Since 2001, this team has become active in providing review and advice on abuse liability data and consultative to individual pharmaceutical companies on strategies for the submission of drugs with central nervous system (CNS) effects. To be more involved in the development of abuse liability strategies, individual pharmaceutical companies formed the Cross-Company Abuse Liability Consortium (CCALC) to provide the opportunity to support scientific discussion with CSS and other scientists with the goal of having input in future regulatory decisions. Transcripts that summarize the discussions of these face-to-face discussions are available. In Anonymous 2006, the EMA published a guidance on preclinical abuse liability testing and in 2012 the FDA published a draft guidance on preclinical abuse liability testing. The topic of abuse liability potential of new drug candidates is also discussed in the M3 document (2009) titled "Nonclinical safety studies for the conduct of nonclinical trials and marketing authorization for pharmaceuticals."

The CCALC has participated in several interactive discussions with the CSS. The proceedings from these meetings are publicly available for anyone interested.

3 Regulatory Expectancies

The flowcharts from both the EMA and the FDA (see Figs. 2 and 3) documents offer direction to drug developers on how to identify the need for specific evaluations to support recommendations to the DEA for scheduling when a new compound is approved for clinical use. The first step in the process is the identification of central nervous system activity as the prerequisite for conducting these studies. The identification of compounds with CNS activity can easily be determined from preclinical data available early in pharmaceutical compound discovery and development. The most obvious indicators of CNS activity are related to indication/therapeutic class for the compound, proposed mechanism of action, binding assay data, cerebrospinal fluid (CSF) and brain circulation of the compound, microdialysis, and brain imaging. The activity of a compound to produce functional changes in traditional CNS function assays such as CNS signs, locomotor activity, electroencephalography (EEG), convulsion, or brain imaging changes would all be

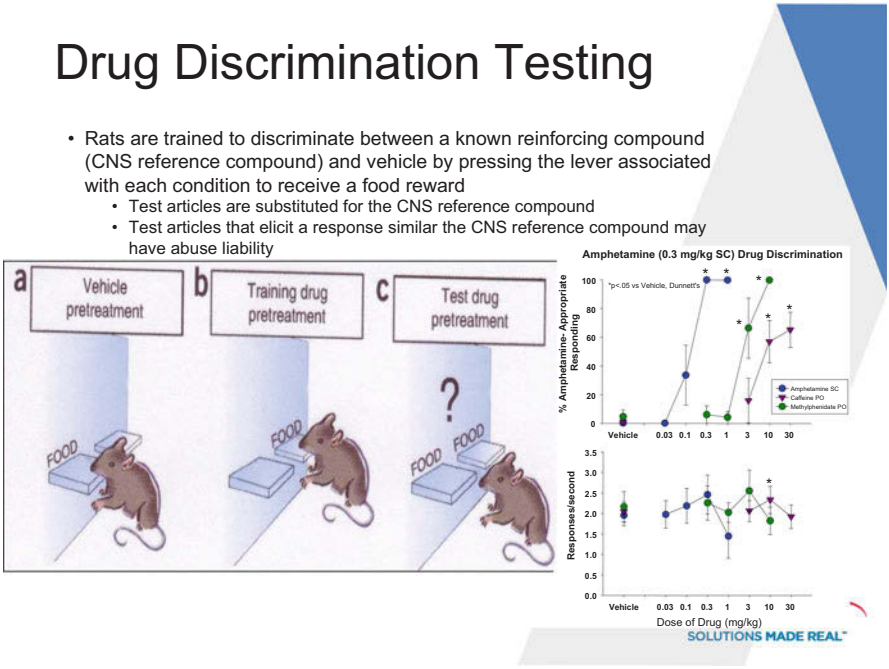

Fig. 2 Illustration of drug discrimination paradigm (*left panel*). On the *right side* of the figure, data are presented for the generalization of caffeine and methylphenidate to an amphetamine training cue of 0.3 mg/kg s.c.

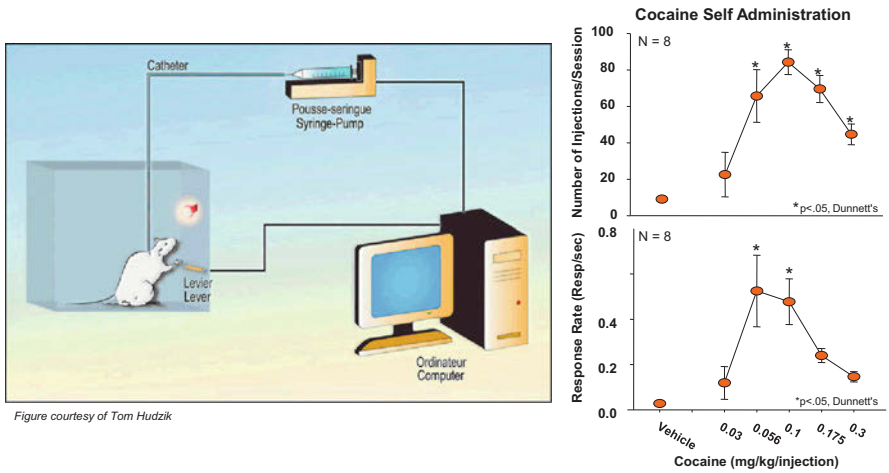

Fig. 3 Illustration of the self-administration paradigm (*left panel*). The *right panel* shows data for cocaine in self-administration

indicative of the directive for abuse liability evaluations. Compounds with binding affinity to know receptors associated with abused compounds will be scrutinized most. These receptors include dopamine, norepinephrine, serotonin, γ-amino butyric acid (GABA), nicotinic, acetylcholine, opioid, NMDA, and cannabinoid receptors. It should also be acknowledged that some compounds that are abused by humans do not produce positive profiles in the traditional preclinical abuse liability models. Examples of this discordance are anabolic steroids that are used in body-building to improve performance in various sports. They are not abused in animals but widely abused in the young sports-focused population. Another example would be new obesity-/diet-reducing drugs that act peripherally on the gut rather than the CNS. Although peripherally active, these compounds would be abused by some human populations but would not have traditional preclinical abuse profiles.

The timing of the conduct of the abuse liability assessments should occur mid- to late Phase 2 of clinical development when the upper therapeutic dose has been identified and the plasma level at C_{max} in humans is characterized. Abuse liability assessments will require preclinical testing at doses up to three times the highest clinical blood level at C_{max} for the highest intended clinical dose if the compound can be dosed at that level without toxic consequences. The clinical information on the upper therapeutic dose is not typically available until late in Phase 2 so studies conducted earlier have the risk of either not being sufficiently complete for scheduling recommendations or testing may be conducted at heroically high doses that

are not required. Any assessments conducted earlier, without an understanding of the therapeutic doses, may require replication at higher doses or may influence scheduling decisions at a dose range that is higher than required. If the drug development company is interested in screening compounds to make strategic decisions about the continuation of molecule development, special consideration of an approach that does not interfere with the required data that will be collected in Phase 2 under structured conditions should be planned. A single approach for screening has not been accepted across laboratories but is a topic that will receive more discussion in the future. Some companies have internal strategies for screening that generate data that is used to make go-no-go decisions about continued drug development. Compounds with a high risk for abuse potential may be dropped from continued drug development efforts.

4 Preclinical Studies Required

Abuse liability assessments are based on three preclinical study types (see the FDA guidance, 2013). The three study types included are a dependence/withdrawal study, a drug discrimination study, and a self-administration study. Data from the three preclinical study types will be coupled with data from the human abuse evaluations to provide a perspective on potential scheduling at the time of the NDA submission and compound registration. An ideal preclinical evaluation should have a strong predictability of human abuse. Of the three models, positive effects in both the dependence/withdrawal study and the drug discrimination studies are not directly related to the question of abuse. Positive effects in the third study type in this package, the self-administration paradigm, is the best predictor/correlate to human abuse (O'Connor et al. 2011; Horton et al. 2013). The overall concordance between the rodent data and human abuse is estimated to be 95 % and the concordance between the primate self-administration and human abuse is estimated to be 97 % (O'Connor et al. 2011). These concordance rates are very similar for rodents and primates supporting the position that rodents are sufficiently predictive to be used in most circumstances for predicting the human potential for abuse. Data from dependence/withdrawal studies can be used to address discontinuation patterns for compounds without abuse potential. There are non-CNS drugs that are not abused that have positive profiles in dependence/withdrawal preclinical and clinical models. Examples of this profile are antihypertensive drugs and SSRIs. Neither type of compound is scheduled but these compounds do have discontinuation profiles. With antihypertensive compounds, the rebound hypertension can be clinically significant if the tapering of the drug use is not applied. Due to the implications for clinical patient management and new drug labeling, it has been suggested that all development compounds should be evaluated in preclinical dependence/withdrawal paradigms and not just CNS compounds. A positive effect in the dependence/withdrawal investigation is not sufficient for scheduling but rather becomes one piece of data that contributes to an integrated recommendation on scheduling.

5 GLP Requirement

The FDA draft guidance (2013) states that "The good laboratory practice (GLP) principles described in the guidance for industry S7A Safety Pharmacology Studies for Human Pharmaceuticals (ICH S7A) and in FDA regulations, 21CFR part 58, apply to abuse potential studies in animals." The S7A guidance indicates that the three core required studies on cardiovascular, central nervous system, and respiratory functioning are to be conducted GLP and all other tier two studies or issue resolution studies should be as GLP compliant as possible. Most tier two studies and issue resolution studies are rarely 100 % GLP compliant. Investigators should use characterized material, determine dose formulation concentrations and plasma levels for relevant doses delivered, document the operation of equipment, and have a detailed protocol with delineated protocol amendments to permit reconstruction of the study. Total compliance with GLP principles is not required for these preclinical assessments.

6 Strategic Choices for Preclinical Abuse Potential Studies

6.1 Species Selection

Several decisions other than doses for the conduct of these studies are required before the actual data collection. One of the most important decisions is the choice of species for conducting the evaluations. Although most industry studies are conducted with rats, the justification for species choice should be considered. There may be legitimate reasons for selecting a non-rodent species for these studies. A recent paper discusses when primates may be a better choice (Weerts et al. 2007). Both the EMA guideline and the FDA guidance recommend the rodent as the preferred species for these evaluations. The alternative to the rodent would be the primate where there is a sufficiently large database for placing a perspective on results of primate evaluations with a novel compound. In preclinical drug development, the most detailed data sets are obtained in the conduct of the toxicology studies where two species are required. The rodent and a large animal species are typical but most frequently the dog is the choice for a second large animal species based on cost and availability. The dog has rarely been used for abuse liability assessments; therefore, if the rat is not sufficient for these studies, then the studies would be conducted in primates due to a larger database for comparison. The primate actually has a higher concordance rate with the human abuse of known compounds but this higher concordance rate is not sufficient to require that primates are always used in evaluation. There are some questions that can direct the decision-making about species selection all based on the knowledge about the compound under consideration for registration as a new drug. The first question deals with the mechanism of action and the conservation of receptors in the rodent to be reassured that the rodent self-administration evaluation will reflect the action of the compound in humans. The second question is about the similarity of the

pharmacokinetic parameters in the rodent and the large animal used in the toxicology studies. If these PK profiles are widely divergent, with respect to C_{max}, $T_{1/2}$, and the metabolite profile, then probably the primate is the better test subject. The third question is: are all CNS active metabolites that circulate at 10 % or higher of the level of the parent compound in plasma represented in the rodent? If not, then CSS would recommend evaluation of each metabolite alone in the abuse liability package which can be a costly requirement where sufficient quantities of the metabolite would have to be synthesized to conduct these evaluations. Conducting primate studies might be a more cost-efficient approach in this situation. A fourth question for consideration involved whether the toxicology profiles are similar for the rodent and the large animal. Clearly, a maximum tolerated dose for a compound will affect the ceiling level of dosing for the abuse liability studies. A fifth question of some importance is the sufficiency of the scientific database on abuse liability for compounds with similar actions in the rodent. A good example of compounds that are more difficult to evaluate experimentally in the rodent are sedatives since better predictability has been reported with the primate than the rodent. When working with anesthetics and sedatives, the primate may be the best choice for designing abuse liability studies from the beginning. The CSS will expect some justification for species selection for the study package, so organizing the information on these questions will help to explain the selection.

6.2 Dose Selection

The doses selected for investigation in an abuse potential package are based on the upper range of the intended therapeutic dosing window. The preclinical studies should approximate a dose that produces a blood level three times the blood level produced at the upper end of the human therapeutic dose. This upper dose in the preclinical studies assumes that toxicity or significant behavioral effects would not prevent the escalation of the dose to this upper range. Behavioral disruption that interferes with the ability of the animals to make lever presses in the drug discrimination and self-administration studies definitely limits interpretation of the data generated with these models. Typically, the mid- and low doses for these studies are within a log range for these types of studies, but the doses are selected to cover a range of effects. Pretreatment times for testing in these models should also take into consideration of the C_{max} and time course for these selected doses. This information is usually available from earlier pharmacology and toxicology studies to support the development of the compound. Doses that significantly interfere with operant-level pressing are unacceptable for the drug discrimination and self-administration paradigms.

6.3 Comparator Compounds

Other questions that should be answered to support the design of an abuse liability package of studies includes what comparator compounds will be used in the studies and what training drugs will be used for the drug discrimination and self-administration studies. Whenever possible, comparator compounds should be consistent across all the studies and representative of the scheduled therapeutic indication intended. There may be situations where more than one comparator compound is useful but the FDA has discouraged the use of a negative comparative compound as the vehicle condition should be representative of the negative profile. An example about how comparator compounds might be selected can be taken from the work on atomoxetine (Upadhyaya et al. 2013). Atomoxetine (a norepinephrine reuptake inhibitor) is approved for the treatment of attention deficit disorder (ADD). The comparator compounds used in these studies were amphetamine and methylphenidate, both Schedule 2 compounds for the same indication.

6.4 Route of Administration

The route of drug administration for these studies differs depending on the study type. For the drug dependence/withdrawal study, the intended therapeutic route of administration is used. Data from both the toxicology and toxicokinetic studies provide the basic information on adverse effect levels and the plasma levels produced by the delivery of the compound by the therapeutically relevant route. The route for the dependence study would be oral, to be consistent with the most common route of administration for new drugs, and the drug would be administered once a day.

The drug discrimination study requires a well-defined drug cue with a reasonably short pretreatment time to support the behavioral performance on the task. Subcutaneous and intraperitoneal injections are most common permitting pretreatment times of 15–60 min prior to behavioral testing on the discrimination task. Other routes of drug administration that have been used in drug discrimination studies include oral (Shelton and Grant 2001), smoking (Newman and Carroll 2006), and inhalation (Bowen 2006; Shelton 2007; Shelton and Grant 2001; Shelton and Nicholson 2013; Shelton and Slavova-Hernandez 2009; Weiss et al. 1979; Wood et al. 1977). The effects produced by the drug for evaluation may not be available; therefore, a pilot study to understand clinical signs and blood levels of the compound may be required prior to initiating the drug discrimination study. Other routes of drug administration can sometimes be used in these studies, but divergence from traditional approaches may require extensive model development prior to the execution of the definitive study and is not preferred by the CSS.

7 The Dependence/Withdrawal Model

Typically, the dependence/withdrawal study requires rodent exposure by the intended clinical route for 2–4 weeks and then the characterization of the period when drug administration stops. The duration of drug exposure and the duration of the withdrawal phase are determined by the pharmacokinetics of the compound. The compound which is given once a day for toxicology profiling is usually given for 2 weeks with a 7–10-day withdrawal phase. The treatment groups examined in the study includes a vehicle control group; a low, mid-, and high dose of the new compound; and a single-dose comparator group. The selection of a comparator compound is based on a known compound from the same drug class or having a similar pharmacological profile that is scheduled. An additional negative comparator is not required. The standard design would include the assessment of clinical signs, locomotor activity, body temperature, body weight, and food intake before the initiation of dosing, at least on the last day of dosing, and daily for most of the assessments. Clinical signs should be collected more than once a day to adequately capture the behavioral changes that might occur during withdrawal. One week is typically sufficient to characterize a return to a normal state, but if all functions are not normal, then the withdrawal period should be extended. Additional assessments during the withdrawal phase of the study might be added for individual compounds with specific pharmacology. If a compound affects blood pressure or seizure threshold, for example, then blood pressure and EEG or convulsion rate might be included in the withdrawal phase. Compounds with a long duration of activity or one that is given twice per week would require a longer dependence phase and longer than 1 week to characterize withdrawal. The scientific literature offers multiple examples of comparator compounds that can be selected for a comparator treatment group.

8 The Drug Discrimination Model

The second model, the drug discrimination model, depends on the animal's ability to detect drug effects produced by drug exposure prior to the testing session to control responding during the session. Figure 2 illustrates the behavioral testing environment and a graph of data generated in this model with amphetamine as a training drug and the generalization of caffeine and methylphenidate to the training drug. The testing chamber is outfitted with two response levers. Responses on one lever are reinforced when a drug is given before the session and responses on the other lever are reinforced when a vehicle or saline is administered prior to the session. Primates, rats, and humans readily learn to distribute their responses in order to maximize the delivery of reinforcement. Scientifically, this technique was first used to identify drugs with similar pharmacological effects. The animals do learn to respond specifically to the actual dose delivered prior to the session since responding on the drug appropriate level is proportional to the dose of the drug administered. Animals are trained to respond in the assay to a training compound.

When responding on the drug, lever is stable and reliable testing of novel compounds can be conducted. The inclusion of data from this model in the abuse potential model is to explore whether the novel compound is similar to known, abused compounds or to identify if the novel compound produces a reliable discriminate effect. Humans typically abuse drugs that produce effects that the individual can identify and describe. Most of the compounds that have been successful discriminative stimuli in this model are predominately CNS active compounds. Although most of us could discriminate some peripherally active compounds like atropine, which produces dry mouth, or a drug that would increase heart rate, peripherally active compounds have not been used as frequently in this model and are not as easily conditioned as discriminative stimuli. Multiple training drugs or multiple drug discrimination studies maybe appropriate depending on the proposed mechanism of action for the novel compound. This assay is also not sufficient for recommendations on scheduling, but these data contribute to the integrated assessment. Many different drugs and CNS mechanisms have been explored with the drug discrimination model in the last 50 years. See excellent reviews for different mechanisms that have been explored in the discrimination model. The reader interested in specific compounds and training doses is referred to the scientific literature or one of the earlier symposiums on the application of this technique in pharmacology (Colpaert and Rosecrans 1978; Colpaert and Slangen 1982).

9 The Self-Administration Model

The self-administration model is the third model in the abuse potential package. This model is based on a within-subject design that is applied to determine if animals will work to get access to a drug (See Thomsen and Caine, 2005). Figure 3 depicts the testing setup and provides some data on responding under the testing paradigm for cocaine, a highly preferred drug for both humans and animals. All data points on the graph were collected from the same animals rather than different groups of animals; thus, this design is a within-animal design. Animals are surgically prepared with an intravenous catheter. Lever responses, 5–10, in the operant chamber produce the delivery of a small drug bolus that is delivered through the intravenous catheter. Animals are initially trained to respond for a drug with known reinforcing characteristics. Cocaine is the drug used most frequently since animals are known to respond about 50–60 times/h for a small injection of cocaine. Other drugs such as morphine or midazolam will also support stable baselines of responding in this test situation. Once responding for a compound with known effects is stable, then other drugs can be substituted for the training drug. The standard preclinical test session is 1–2 h in duration but that can be extended, if needed, for assessing drugs with longer durations of activity. The most difficult aspect of this evaluation is maintaining catheter patency and surgical repair since animals are repetitively tested across several months. A dose response curve would be generated in the self-administration model for the novel compound and at least

one comparator compound. A comparator compound should be scheduled and have a history of self-administration. This model is similar to the setup of a Demerol (meperidine) pump in the hospital postsurgical suite. Once recovered from anesthesia, postsurgical patients can self-administer drug for pain relief by pressing a button. Patients usually require less pain relief medication when they can control the delivery of the medication. For reviews of data collected with the self-administration model, see Balster and Bigelow (2003) or O'Connor et al. (2011).

When a new drug is self-administered, it is necessary to determine the reinforcing quality of the new drug. Two approaches have been used to determine how hard animals will work for the compound. One approach is to test under progressive ratio conditions where the number of lever presses required for a single drug injection increases until the animal quits working (Roberts and Bennett 1993). The quantitative point where they quit working is called "the breakpoint." The breakpoint can be used to make comparisons across drugs. In the human situation, this is similar to how much one would be willing to pay to get a drug or how hard one would work to get the drug.

A second approach for understanding the value of a reinforcing drug is to use a choice paradigm. The Gasior et al. (2005) paper is an example of the use of the choice paradigm in the lab. An animal has the opportunity to choose between food and a drug as a reinforcer. The index effect tracks when responding shifts from responding for drug to responding for food.

The greatest strength of the self-administration is that the data addresses the question of will an animal take a drug and the strong predictability to the human abuse situation (O'Connor et al. 2011). The weaknesses of the model are that it has both false-positive and false-negative profiles, requires intravenous drug administration, and is dependent on surgically prepared animals for stable data collection.

10 Integration of Data

These data are integrated to provide a recommendation about the potential need to schedule the new compound for clinical use. The US and the European flowcharts for the conduct of studies that will be used to provide an integrated profile for the regulatory agencies are presented in Figs. 4 and 5. These two flow schemas are very similar. The primary exception is that European regulators expect drug discrimination *where the novel drug is the training drug*. The justification for this study is that a novel mechanism compound may not have an appropriate comparator for conducting drug discrimination. If the novel compound produces a discriminable stimuli, that would be an initial indication that further work would be required. The problem with actually applying this approach is it is difficult to identify how much training would be required to demonstrate that the animals cannot learn to discriminate the novel cue. Is a month, 6 months, or 6 years required? It is difficult to demonstrate that the null hypothesis or no effect is in action. Comparisons with known abused drugs that are used as the training drug in these paradigms will be influential in the decision reached. After the completion of the human data

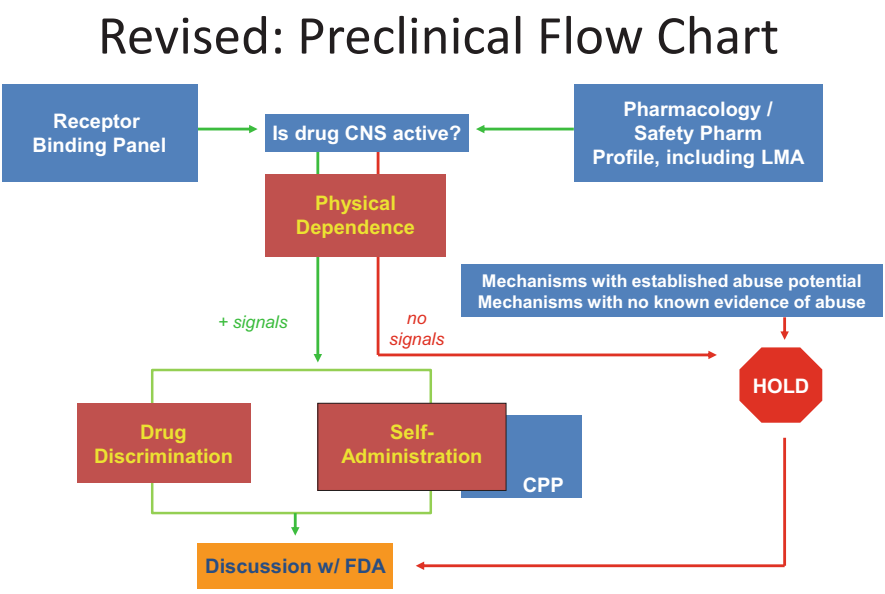

Fig. 4 CSS/FDA flowchart for the conduct of specific abuse potential assessments

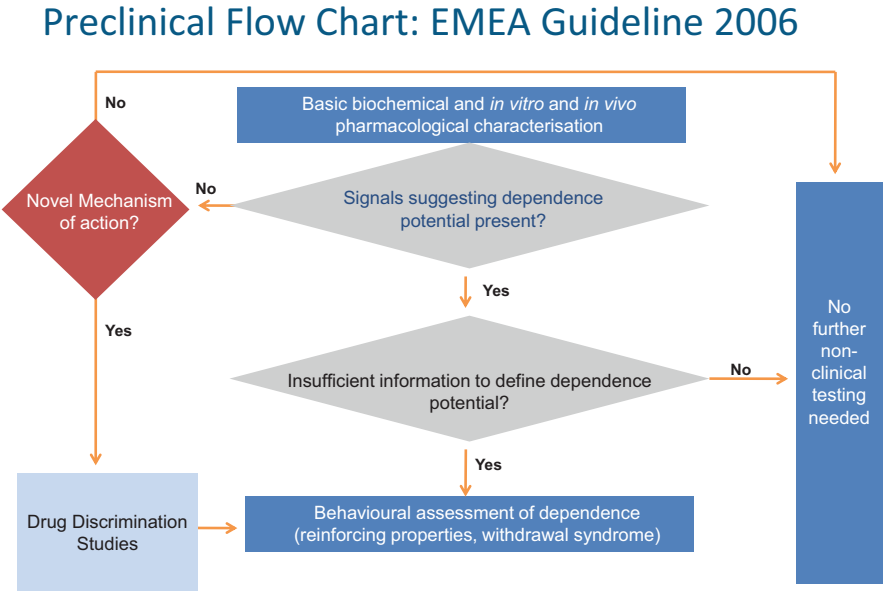

Fig. 5 EMEA flowchart for the conduct of specific abuse potential assessments

assessment, the human and animal data will be considered as a whole package. The data are weighted in several ways. First, the human data always weighs heavier than

animal data for reaching a final decision. Among the three animal assessments, the self-administration assessment is most valued as data from this paradigm directly addresses whether the compound will sustain responding for the self-administration of the compound. As indicated in the earlier section on self-administration, positive data in the self-administration paradigm should be put into perspective by testing in progressive ratio or in the choice situation that provides information about the potency of the novel reinforcer. The regulatory decision around the scheduling of a new compound is coupled with the need to monitor post-marketing data which can alter the decision made at the time of approval if necessary.

10.1 How Are the Data Used?

The data collected in the three required preclinical assays and the clinical data collected will be used by the CSS/FDA to make a recommendation with consultation from the National Institute on Abuse Liability to the Drug Enforcement Agency (DEA) about scheduling at the time that the NDA is submitted. The preclinical and clinical data will be integrated by the company seeking registration of the compound in a draft 8-factor document. The FDA will edit the draft 8-factor document, much like occurs with a drug label to be finalized. Figure 6 lists the 8 factors that must be discussed in this document. The document homogenizes clinical and preclinical abuse potential information with the competitive therapeutic landscape. The parameters that reflect the preclinical data are indicated in Fig. 6.

The preclinical data can also impact the design and breadth of the clinical abuse liability package that will be expected. In addition, the data from the dependence

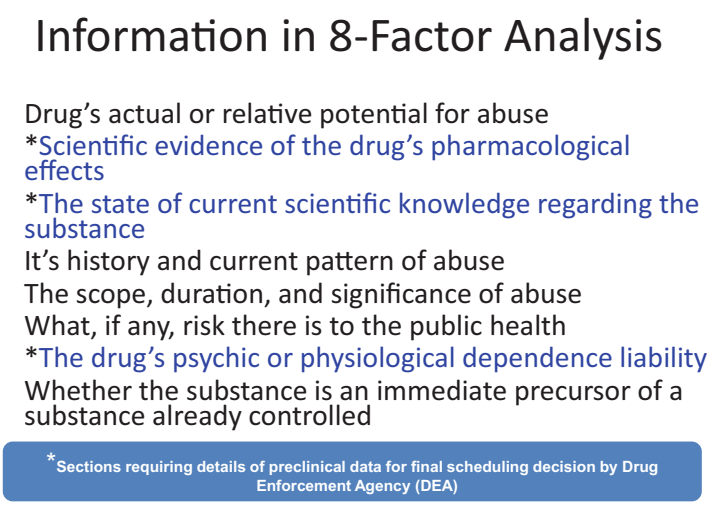

Fig. 6 A list of the specific sections included in each of the 8-factor document headings. Sections 2, 3, and 7 are based on preclinical data

study will support clinical recommendations on drug tapering for medication changes. This information is included in the label for the marketed compound and will be available for physician advice.

10.2 What Is the Future in Preclinical Abuse Liability Testing?

The pharmaceutical industry has established avenues for interaction with the Controlled Substance Staff through participation in face-to-face meetings and the joint organization of symposium at scientific meetings to encourage the discussion of topics of common interest. This interaction is expected to continue in the future with interactive involvement in new models and the implications of data generated. Currently, planning is ongoing for a face-to-face meeting between the CCALC and the FDA for April 2015.

The development of some of the secondary models such as conditioned place preference and electrical brain stimulation which do not require intravenous drug injections will continue to occur (for complete descriptions of the conditioned place preference paradigm and validation data, see Bardo and Bevins (2000) or Cunningham et al. (2006, 2011); for a complete description of the electrical brain stimulation model, also see O'Neill and Todtenkopf (2010) or McBride et al. (1999)). A better understanding of the predictivity of these models and correlation with the traditional self-administration model will be required for these models to assume a mainstream position in abuse potential assessment. The self-administration model's predictive correlation to abuse potential in humans has been well characterized but new models will have to be characterized and published to have equal impact in future abuse potential testing.

Other considerations that will shape the future include a better understanding of potential screening approaches for abuse potential, how to handle large molecules (see de Zafra 2014), and the development of a broader database for resolution of drug development strategies and the selection of comparator compounds.

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Overview of Respiratory Studies to Support ICH S7A

Michael Stonerook

Contents

Abstract

Tests of pulmonary function are useful tools for evaluating the potential for compounds to produce toxicity affecting the pulmonary system. Insults to the pulmonary system (i.e., due to drugs, biologics, toxins) can cause detectable dysfunction through multiple mechanisms. Manifestation of the response to insults will depend on the component(s) involved and the compensatory mechanism(s) initiated. The purpose of this chapter is to introduce the concepts of pulmonary testing as it is applied to the preclinical evaluation of pharmaceutical test articles. The topics will include the techniques and methods that have been developed for use in nonclinical (animal) subjects and the parameters that are routinely measured.

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Keywords
Blood gases • Compliance • ICH S7A • Oscillometry • Oxygen saturation • Plethysmography • Pneumotachograph • Resistance • Respiratory inductance plethysmography (RIP) • Respiratory rate • Tidal volume

1 ICH S7A: Why Conduct Respiratory Safety Pharmacology Studies?

The cardiovascular, respiratory, and central nervous (CNS) systems were selected by the regulatory guidances since they represent vital functions where acute dysfunction can lead to serious adverse events. The function of the respiratory system is interesting among these three systems in that it is tightly integrated with the neural control from the CNS for ventilation and reflexes and the distribution functions for gases by the cardiovascular system.

Based on the guidance provided in the ICH S7A document (ICH 2000) and the FDA guidance for industry (FDA 2001), respiratory safety pharmacology studies should include *respiratory rate and other measures of respiratory function* (e.g., *tidal volume or hemoglobin oxygen saturation*). The term respiratory function generally refers to a number of endpoint parameters of the pulmonary system from ventilation (rate, tidal volume, minute volume) to airflow within the tracheobronchial tree (resistance, airflow, flow durations), to the ease of lung motion and filling (compliance, elasticity), and finally to alveolar gas diffusion, all of which result in a homeostatic balance of the dissolved gases in the blood (i.e., PCO_2, PO_2, and oxygen saturation).

There are numerous classes of drugs that have been documented to produce altered pulmonary function including chemotherapeutics, analgesics, antibiotics, and anti-inflammatories among others (Gad 2004). Using the PharmaPendium™ database, a search on drug-induced respiratory disorders showed that the percent of total drug products associated with breathing abnormalities (ventilatory alterations), airway obstruction, or lower respiratory tract disorders were 40 %, 29 %, and 31 %, respectively (Murphy 2014b). Although not common, acute treatment with drugs can cause pulmonary edema or pneumonitis, whereas, in repeat-dose studies, alterations in the fibrous network, surfactant disruption, or pulmonary congestion are more common (Erasmus et al. 2002).

In most respiratory safety pharmacology assays, respiratory rate, tidal volume, and their mathematical product, minute volume, are the primary current parameters assayed. When respiratory study endpoints were assessed in the Safety Pharmacology Society's best practices survey (Lindgren et al. 2008), 100 % of respondents ($N = 64$–66) measured respiratory rate and 98 % measured tidal/minute volume; however, only 39 % measured compliance/resistance and only 16 % measured additional endpoints including blood gases, peak inspiratory and expiratory flow rates, inspiratory and expiratory time, enhanced pauses, lung sounds, and pulse

oximetry. The value of evaluating resistance and compliance as endpoints has been reviewed for the value of these parameters to characterize acute drug-induced effects on the lung (Murphy 2014a, b).

Changes in respiratory function can result either from alterations in the pumping apparatus including nervous and muscular components that control the pattern of pulmonary ventilation or from changes in the mechanical properties of the gas exchange unit consisting of the lung with its associated airways, alveoli, and interstitial tissue (Murphy 2002). Defects in the pumping apparatus and reflex-related alterations can change the breathing pattern and can be tested noninvasively in conscious animal models. Defects in mechanical properties of the lung can result in obstructive or restrictive disorders which often can also be detected by noninvasive lung function parameters but can be better evaluated by invasive lung function tests and pulmonary maneuvers in anesthetized animals due to their higher sensitivity and specificity.

The rat remains the most widely used species (Lindgren et al. 2008) for the conduct of respiratory safety pharmacology studies while presenting a respiratory response sometimes different from humans (Legaspi et al. 2010). However, other species are also viable candidates for use in respiratory safety pharmacology studies. The minipig, dog, and primate have been evaluated and are considered to be a valid alternative to other non-rodent species for cardiovascular and respiratory safety pharmacology studies (Authier et al. 2008, 2009, 2011; Truchetti et al. 2014).

The typical study design for such a study involves a single administered dose to each of three dose-level groups (and a control group) where $n = 4–8$ animals in each dose group. Data is collected for a baseline period prior to dose administration and then subsequently post-dose for several hours and may include a second data collection targeting the 24-h post-dose period to evaluate the resolution of any effects noted. Because of the nature of acute dosing and data collection, only compounds that produce rapid pharmacological effects will likely have detectable signals. Compounds that may take hours to days to manifest effects either as structural changes or as a result of repeated dosing are not typically identified during the stand-alone safety pharmacology study; however, it may be detectable if the respiratory study is incorporated as an element of a longer-term pivotal repeat-dose study (Redfern et al. 2013).

2 Lung Anatomy and Physiology

The principal function of the respiratory system in mammals is gas exchange. The functional unit of the lung is the pulmonary acinus and associated alveoli. This requires the presence of a membrane with a very large surface area, but with minimal thickness. The barrier that separates the air surface of this membrane

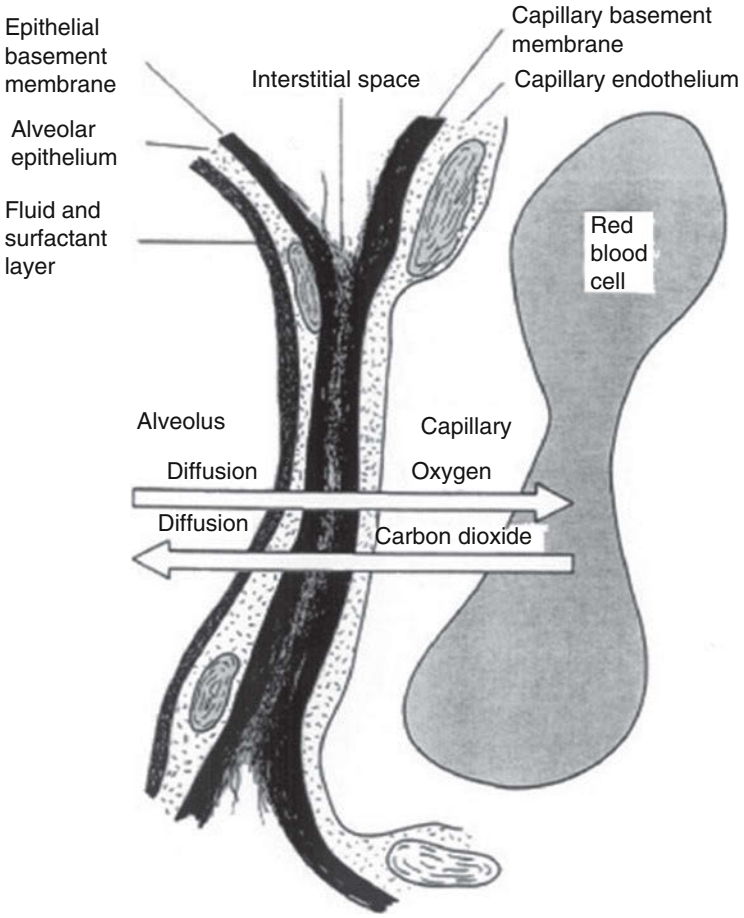

Fig. 1 Schematic representation of the blood-gas barrier at the level of the alveolus-pulmonary capillary

from the large vascular bed associated with it is limited in thickness by the requirements for gas diffusion (Fig. 1).

The airways and lungs exhibit a significant amount of species diversity in composition of the cellular elements and organization of the tracheobronchial structures and the lungs themselves (Tyler and Julian 1992). These variances have important consequences when considering an animal species for the evaluation of drug effects, particularly compounds that may be administered through the inhalation route. See Table 1 for details regarding a comparison of lung structure in nonclinical animal species to that found in humans.

Table 1 Comparison of lung structures between species

	Human	Macaque	Dog	Rodent, rabbit	Pig
Pleura	Thick	Thin	Thin	Thin	Thick
Interlobular connective tissue	Extensive	Little	Little, if any	Little, if any	Extensive
Non-respiratory bronchiole	Several generations	Fewer generations, commonly only one	Fewer generations	Several generations	Several generations
Respiratory bronchiole	Several generations	Several generations	Several generations	Absent or single short generation	Absent or single short generation

Modified from Tyler and Julian (1992)

3 In Vivo Assays: Techniques and Endpoints

Insults (due to drugs, biologics, or toxins) can cause detectable changes in function through multiple mechanisms depending on the nature of the insult, timing, route of delivery (air or blood), duration of insult, and the initial respiratory health of the animal. The manifestation of the dysfunction will depend on which components of the respiratory system are affected and the compensatory mechanisms initiated. The pattern of dysfunction detected may reflect the primary response or may reflect the compensatory changes to the primary response.

Studies of the diseased lung have provided a basis for two broad categories of impairment based on the type of dysfunction that are termed restrictive or obstructive. Restrictive dysfunction (or restrictive ventilatory defects) would include diseases which affect the lung parenchyma or pleura and interfere with normal expansion of the lung, i.e., increase lung stiffness. Obstructive dysfunctions are characterized by an interference with normal airflow through the airways. In many cases, these dysfunctions may not produce a change in ventilatory activity due to the large dynamic reserves of the lung and cardiovascular system to maintain ventilation-perfusion matching (Costa et al. 1992).

Respiratory safety pharmacology studies can be conducted on a series of tiers that allow for progressive interrogation of the system as abnormalities are noted. The first tier of assays is, at a minimum, conducted to evaluate ventilatory functions including respiratory patterns (rate, tidal volume, and their product which is minute volume), resistance of airflow within the large and/or small airways, and the compliance of the structure of the lung. As noted previously, in most respiratory safety pharmacology assays, respiratory rate, tidal volume, and minute volume are the primary parameters assayed for drug safety evaluation (Lindgren et al. 2008); however, there is sound scientific basis for the inclusion of resistance and compliance assays (Murphy 2014a).

The validity of rate and volume measurements alone to evaluate respiratory changes has been shown to be in question. Studies have demonstrated that two- to threefold increases in airway resistance using an intravenous infusion of the bronchoconstrictive agent, methacholine, does not cause ventilatory changes in the rat, dog, or monkey (Murphy 2014b). Given that the ventilatory parameters of rate and volume may be inherently insufficient for a complete evaluation of respiratory changes, the addition of other endpoints such as times and flows of inspiration and expiration can provide further detail (Legaspi et al. 2010). For example, a selective increase in inspiratory time or decrease in mean inspiratory flow (tidal volume/inspiratory time) is indicative of a decrease in respiratory drive (Remmers 1976) while a selective increase in expiratory time or decrease in expiratory flow can be indicative of airway obstruction (Glaab et al. 2002). In addition, the presence of an end-inspiratory pause or end-expiratory pause has been shown to be indicative of upper airway sensory and lower airway irritant receptor activation, respectively (Ferguson et al. 1986).

An additional consideration for the endpoints to be examined is related to the timing of the insult, whether acute or chronic, as this will produce different responses based on the nature of the injury and the ability of the cardiorespiratory system to respond to maintain homeostatic balance (see Table 2 for details).

Table 2 Response of the respiratory system to insult

Peracute responses: neurally/receptor mediated	
Mechanism • Bronchoconstriction ↓ Relaxing factors ↑ Contracting factors • Mucus secretion • Ventilatory events Cough/sneeze/laryngospasm Apnea, tachypnea, hyperpnea	Endpoints • ↑ Airway resistance • ↓ Dynamic compliance (Cdyn) • ↑ Work of breathing • Δ Breath sounds • Δ Distribution of ventilation • Δ Rate, tidal volume, minute ventilation • Δ Arterial blood gases
Acute responses: changes in structural integrity	
Mechanism • Δ Endothelial permeability • Δ Epithelial permeability • Bronchoconstriction – Hypersensitivity	Endpoints • ↑ Airway resistance • ↓ Dynamic compliance (Cdyn) • ↓ Static compliance (Cstat) • ↑ Work of breathing • ↑ Lung water (edema) • Radiographic changes • Δ Lung cell populations • Δ Arterial blood gases
Chronic responses: structural changes ± repair changes	
Mechanism • Fibroproliferation – Fibrosis • Alveolar destruction – Emphysema • Airway remodeling • ↓ Clearance promoting infection	Endpoints • Δ Cstat, Cdyn • Δ Forced volume/flow maneuvers • ↑ Airway resistance • ↑ Work of breathing • Radiographic changes • Δ Arterial blood gases • Δ Lung cell populations

4 Assays for Ventilatory Function

Change in the functional status of ventilation is determined by measuring respiratory patterns which should include, at a minimum, the endpoints respiratory rate (frequency), tidal volume (depth), and minute volume (or minute ventilation or expired minute volume). By monitoring the frequency and depth of the pumping apparatus, the effects of drugs on total pulmonary ventilation (i.e., respiratory stimulation or depression) can be established.

4.1 Plethysmography

4.1.1 Pneumotachograph

A pneumotachograph (PT) is a device consisting of a resistance element across which a flow occurs which creates a pressure difference between the two sides of the resistance element according to the following relationship:

$$\text{Flow}(F) \times \text{Resistance}(R) = \text{Pressure Difference}(\Delta P)$$

The pressure difference generated by a PT is directly proportional to the flow rate as long as the flow is laminar. Note that the laminar flow has less resistance than when the flow is turbulent. Flows with a Reynolds number (a dimensionless parameter of the airflow) less than 2,000 are considered laminar.

The resistance elements that are used in commercially available PTs can be based on different technologies including fine mesh wire screens, each of which has a uniform hydraulic diameter (Hans Rudolph, Shawnee, KS), bundled small capillary tubes which create pseudo-laminar flow (Fleisch Vitalograph®, Lenexa, KS), or a constriction of the airflow path (as observed with a Venturi-type effect). The PT is connected to a differential pressure transducer that is coupled to an amplifier to measure the pressure difference and is calibrated in units of flow (mL/min or L/min depending on the normal range of the species measured). Data acquisition software is then used to integrate the flow signal with time to provide a volume calculation as well as various waveform morphology calculations such as inspiratory/expiratory time and other characteristics (Fig. 2). Pneumotach can be placed in line with the breathing path of an animal by using a mask in conscious subjects or an endotracheal tube in anesthetized subjects. Alternatively, PTs can be used with plethysmographs (described in next section) to avoid being placed in line with the breathing path. Wire screens have been integrated into the wall of a plethysmograph to create an integrated PT with good response frequencies and this design is commercially available from several vendors for small species such as mice, rats, and guinea pigs.

4.1.2 Plethysmograph (Restrained)

A plethysmograph (from the Greek, *plethysmos*—to increase) is a rigid-walled container into which the animal is placed to allow the measurement of flows and/or pressure changes that can be integrated to report volumes. Plethysmographs

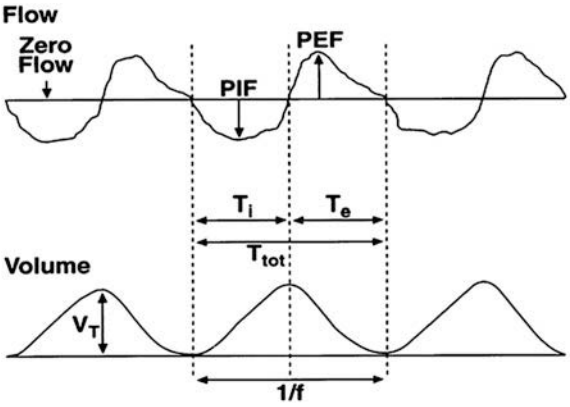

Fig. 2 Functional endpoints of ventilatory patterns in animals. F, frequency (respiratory rate); V_t, tidal volume; T_i, inspiratory time; T_e, expiratory time; PIF, peak inspiratory flow; PEF, peak expiratory flow

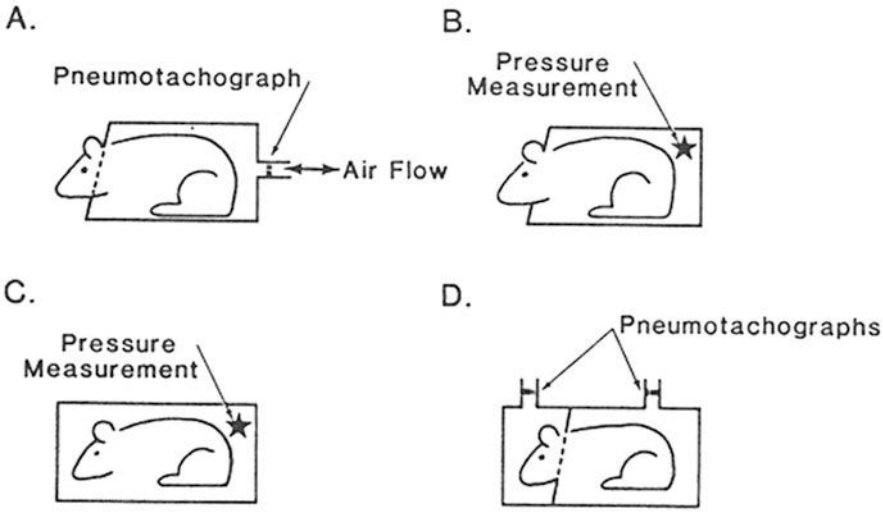

Fig. 3 Schematic diagrams of the different types of plethysmographs. (**a**) Head-out with volume displacement, (**b**) head-out with constant volume, (**c**) whole body, (**d**) "Pennock"-style dual-chamber plethysmograph to measure both airway flow and thoracic volume changes

are commercially available in a variety of designs and are particularly useful for small animals up to rabbits but have been built for dogs and primates.

There are three basic types of plethysmographs (Fig. 3) based on their design to detect airflows or pressure changes: (1) volume displacement, (2) constant volume, and (3) barometric or whole body. In the volume displacement and constant volume designs, the animal's thorax is enclosed and the breathing airflow is to the ambient air, either by a tube or by the head extending through a diaphragm in the wall. In the volume displacement, as the thorax moves with respiration, an equivalent flow is generated from the chamber and across a PT. In the constant volume, the chamber is

sealed and thoracic volume changes are detected as pressure changes within the chamber. For this design, flow is derived from the volume signal that is calibrated to the pressure changes. Due to the technical challenges and risk of artifacts altering the pressure signal, e.g., adiabatic heating of the chamber, the constant volume design is not commonly in use for safety pharmacology evaluation of respiratory parameters.

Modified plethysmographs both in the style of a head-out which incorporates the body but leaves the head exposed to ambient conditions or in the style of a head-dome which incorporates the head but leaves the body exposed to ambient conditions have been developed for larger animals such as dogs and primates.

4.1.3 Plethysmograph (Unrestrained)

In the barometric (whole body) chamber design, the animal is completely contained within the plethysmograph and has no limitation to movement within the chamber. The use of an unrestrained system allows for longer-duration evaluations and repeated evaluations with minimal disturbance to the animals, primarily rats and mice (Delaunois et al. 2009).

During inspiration, it is observed that the box pressure rises, and during expiration, the box pressure falls. The rise in pressure during inspiration is due to the tidal air, as it moves from the box into the lungs, being warmed to core body temperature (~37 °C) and humidified to 100 % relative humidity by the time gas reaches the alveolar spaces. The difference between the external process of the thoracic expansion, which is adiabatic, and the internal process of it, which is isothermic, is an important factor of internal energy change in the whole-body plethysmography method (Chaui-Berlinck and Bicudo 1998).

The larger the tidal volume, the larger the pressure increase, with other factors being equal. On the basis of the original analysis (Drorbaugh and Fenn 1955), the following formula can be used to calculate the pressure increase during inspiration:

$$\Delta P_{\mathrm{box}} = V_{\mathrm{t}}/C_{\mathrm{gas}}\left[1 - \left(T_{\mathrm{box}}/T_{\mathrm{lung}}\right)(\mathrm{Pb} - \mathrm{PH_2O, lung})/(\mathrm{Pb} - \mathrm{PH_2O, box})\right]$$

where V_{t} is the tidal volume; T_{box} and T_{lung} are the temperatures (°K) in the box and lung, respectively; Pb is the barometric pressure; PH_2O box and PH_2O lung are the water vapor pressures in the box and lung, respectively; and C_{gas} is the compliance (mostly adiabatic compressibility) of the air in the plethysmograph (~75 ml cmH_2O^{-1}).

The equations used by Drorbaugh and Fenn (1955) to calculate tidal volume described only events occurring during inspiration. The neglect of events during expiration has been argued to cause a systematic error that may underestimate V_{t} by up to 30 % (Epstein and Epstein 1978), and it was confirmed that the error increases as the ratio of inspiratory duration (TI) to total breath duration (Ttot) increases and as the expired temperature at the nares increases (Jacky 1980).

Although the whole-body plethysmography for unrestrained animals is the most widely used method to assess the respiratory risk of new drugs in safety pharmacology, non-appropriate experimental conditions may mask deleterious side effects

of some substances. Under normocapnia, the ventilatory depressant effects of morphine can be easily evidenced in mice, slightly observed in guinea pigs, and not detected in rats in any day phase. Slight hypercapnic conditions enhanced the responsiveness of rats to morphine but not that of guinea pigs, and importantly, they did not blunt the airway responsiveness of rats to the stimulation and bronchodilation evoked by theophylline (Goineau et al. 2010).

4.2 Respiratory Inductance Plethysmography

Respiratory inductance plethysmography (RIP) utilizes straps containing inductive coils placed around the thorax and abdomen to measure lung volume changes. A continuous, low-voltage electrical current is passed through the inductive coils, and current changes that are proportional to the changes in length of the inductive coil straps are produced by the expansion and contraction of the thorax and abdomen during breathing. The use of inductance methodology allows for the application of more than one band, providing for the ability to assess both thoracic and abdominal movement; this then permits an assessment of phase differences between the two bands. The resulting patterns produced by each band are sinusoidal in nature and may be summed to determine the total volume inspired. The phasic relationship between the abdominal and thoracic bands may also provide an assessment of pulmonary resistance (Hammer and Newth 2009). Inductance plethysmography has been developed for clinical use, particularly in pediatric medicine (Fiamma et al. 2007; Brouillette et al. 1987; Zupnick et al. 1990).

Inductive systems correlate absolute volume changes, as measured by a pneumotachograph (PNT), to a measured difference in diameter of bands traversing the thoracic and abdominal cavities. This system however requires calibration prior to each collection session and complete stabilization of bands following calibration in order to maintain accuracy for the duration of a collection. Moreover, these techniques require great skill and calibrations need to be carefully performed in order to generate consistent data, which could be of disadvantage.

The original designs to incorporate RIP for use in safety pharmacology studies involved the use of external bands and protective shirts/jackets. External RIP has been validated to be an acceptable method for measuring ventilatory parameters in conscious non-restrained dogs (Murphy et al. 2010; Purbrick et al. 2012) and primates (Maucote et al. 2013). The primary advantages of the external RIP systems are that they can be combined with cardiovascular telemetry in order to reduce the numbers of animals needed to evaluate these two systems, they allow for 24+ hour continuous data collection, and they can be incorporated into repeat-dose study designs. The disadvantages of the external system are the maintenance of the band placement over time, the inaccuracy of the volume data that can be the result of poor calibration or band movement, and the time to place animals into the external jacket systems, particularly for primates.

In an attempt to avoid the issues with external systems, a fully implantable system linked to radiotelemetry was developed based on transthoracic inductance

plethysmography (TIP). The telemetry-based device adds an impedance-based biosensor and lead set for the measurement of respiratory function to the standard cardiovascular telemetry device used in large animals. The impedance sensor detects respiratory changes by injecting a low-amplitude, imperceptible, and non-tissue-stimulating (neither skeletal nor cardiac) electrical current across the thorax and measuring the resultant respiratory-induced voltage modulation via leads placed on either side of the thorax. The measured voltage is then converted into electrical resistance resulting in an electrical impedance waveform that is a correlate to respiratory volume. The primary advantage of this system is that it cannot be displaced once implanted. The disadvantage is that the system placement does require surgery and a knowledge of the best lead placement. Several labs have validated these implantable systems and have found them to be valuable for respiratory assessment in dogs (Kearney et al. 2010; Milano et al. 2011) and primates (Ingram-Ross et al. 2012; Renninger et al. 2012).

4.3 Resistance/Compliance

Airway resistance is quantified by measuring the transpulmonary pressure (mouth pressure – pleural pressure) required to achieve a given lung airflow. Lung compliance is quantified by measuring the change in pressure associated with a change in volume. Lung compliance measurement in single-dose safety pharmacology studies may be limited in sensitivity in that marked lung injury affecting the parenchyma or pleura rarely occurs following acute drug treatment or, if injury occurs, it can generally be detected by histopathological evaluation. However, inclusion of lung compliance as an endpoint should be considered when there is concern for acute lung injury following an inhaled dose of drug or when effects, which may not be detected by histopathological evaluation, such as interstitial edema or alterations in alveolar surfactant production, are anticipated (Murphy 2014b).

Methodologies are currently available for measuring airway resistance and compliance in rodents and non-rodents either during spontaneous breathing (dynamic) or using a forced maneuver procedure (flow–volume or pressure–volume curves) that involves a controlled inflation and rapid deflation of the lung to evaluate forced expiratory flows and static or quasi-static compliance. The direct measurement of resistance and compliance typically requires an anesthetized model so that the airflows, pressures, and volumes can be monitored and controlled (Diamond and O'Donnell 1977; Costa et al. 1992; Mauderly 1989).

For the inclusion of dynamic resistance and compliance measurement in safety pharmacology studies, a technique using an implanted pressure lead from a radiotelemetry device was developed in rats (Murphy et al. 1998) and monkeys (Murphy et al. 2001). This technique placed the pressure catheter tip beneath the esophageal serosa within the pleural cavity. This approach allowed the catheter tip to remain outside of the pleural space thus eliminating inflammatory responses that would occlude the pressure signal over time. When coupled with a head-out plethysmograph located above a radiotelemetry receiver, the pleural pressure signal and

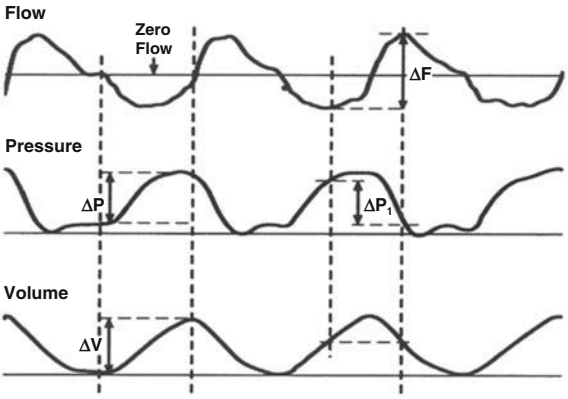

Fig. 4 Functional endpoints for airway resistance and lung dynamic compliance calculations. Shown on the *right side* is the calculation for airway resistance (Raw) where Raw = ΔP/ΔF calculated at points of isovolume. Shown on the *left side* is the calculation for dynamic compliance (Cdyn) where Cdyn = ΔV/ΔP calculated at points of isoflow (typically zero flow). (ΔP—pressure difference, ΔF—flow difference, ΔV—volume difference)

respiratory airflows could be simultaneously recorded and used to calculate dynamic resistance and compliance (see Fig. 4). This technique is subject to issues with the quality of the pressure signal based on the surgical technique (Ewart et al. 2010). The other limitation of this technique is the requirement for restrained plethysmography to collect the airflow signal, thus limiting the duration of data collection possible in the conscious animal.

As an indirect measurement of changes in airway resistance, unrestrained whole-body plethysmography (WBP) has been developed to measure airway reactivity in conscious animals (Lomask 2006). A nondimensional parameter based on a characteristic change in the expiratory waveform of the WBP signal, termed Penh (enhanced Pause), has been used as an indicator of bronchoconstriction. The calculation of Penh is:

$$\text{Penh} = (\text{PEF}/\text{PIF}) \times ((T_e/R_t) - 1)$$

where PEF = peak inspiratory flow, PIF = peak expiratory flow, T_e = expiration time, and R_t = time to relax to 65 % of expiratory volume.

It is of interest to note that the waveform of the WBP is not a true inspiratory or expiratory signal as it is the summed waveform of both the nasal flow and thoracic movement. The separate analysis of both signals can be obtained with an animal in a dual-chamber plethysmograph (Pennock et al. 1979) where the nasal flow signal comes from the head portion of the box and the thoracic signal comes from the body portion of the box. Specific airway resistance can be assessed as the phase angle, or time delay, of the two signals within a dual-chamber plethysmograph. In the WBP where the signals are summed, a phase delay as a result of change in resistance will produce a characteristic wave change that is reflected in the PEF and/or Rt of the

waveform, thus the basis for the correlation of Penh to change in airway resistance which is particularly useful in acute bronchoconstrictive models. Comparison of physiological data generated with the dual-chamber plethysmograph to that generated with the WBP has revealed several differences. While the WBP appeared to give inaccurate measurements of tidal volume, it provided much better analysis of airway reactivity. In contrast, the dual-chamber plethysmograph provided accurate physiological data such as tidal volume and respiratory rate, but provided inaccurate and irreproducible airway reactivity results (DeLorme and Moss 2002).

The technique of using Penh as an index of bronchoconstriction has been supported in mice (Hamelmann et al. 1997; DeLorme and Moss 2002) and has been used in freely moving piglets as a screening index for airway reactivity and pulmonary functional changes in cholinergic and endotoxin challenges (Halloy et al. 2004) and in dogs (Hirt et al. 2008; Talavera et al. 2006).

Several studies have shown that changes in Penh and respiratory resistance sometimes do not correlate (Adler et al. 2004; DeLorme and Moss 2002; Flandre et al. 2003). Investigators have presented mathematical and theoretical arguments that the WBP waveform and parameters derived from Penh are dominated by conditioning, primarily related to ventilatory timing and unrelated to airway resistance (Lundblad et al. 2002; Mitzner and Tankersley 2003); however, it may be the nature of the restrictive changes anatomically and physiologically that limit the sensitivity and specificity of the Penh analysis.

4.4 Airway Oscillometry

Techniques are available that allow for the ability to assess abnormalities in airway mechanics or structure by imposing a small oscillating pressure signal on the airway during tidal breathing (Peslin and Fredberg 1986). The sinusoidal pressure oscillations may consist of a single or a spectrum of frequencies, but must exceed the normal tidal volume frequency. In the clinic, airwave oscillometry is used in evaluation of toxicity and disease conditions (Gube et al. 2009; Frantz et al. 2012).

The alterations in the resulting pressure, volume, and flow signals within the airway can be used to calculate resistive, compliant, and inertial characteristics of the entire system or of anatomical compartments (reviewed by Watson 1992). The techniques have been used for respiratory interrogation in horses (Mazan and Hoffman 2003), monkeys (Watson and Jackson 1985; Wegner et al. 1984), dogs (Jackson et al. 1984; Hoffman 2007), and rats (Jackson and Watson 1982). More recently, the use of oscillometric techniques has been adapted for respiratory safety pharmacology evaluations in both dogs and monkeys (Bassett et al. 2014).

Use of oscillometric techniques to assess airway changes is limited in rodents and small mammals as they typically need to be anesthetized with tracheal intubation to best access the airways. Use of oscillometric techniques in larger species, such as dogs, can be done in conscious animals and only requires acclimation to masks and short-duration restraint making them a viable alternative to plethysmographic techniques.

4.5 Blood Gases and Oxygen Saturation

Monitoring ventilatory parameters cannot generally be used to directly assess the status of the gas exchange unit of the lung. To better characterize the functional consequences of respiratory changes, the impact on blood gas diffusion should be considered. Comparing the relative changes in the partial pressures of arterial O_2 (PaO_2) and CO_2 ($PaCO_2$) is one method for detecting injury at any level that would hinder the diffusion of gases from the alveoli to the pulmonary arterioles as would be caused by interstitial or intra-alveolar accumulation of fluid, reduction in alveolar ventilation by small airways, or the reduction in alveolar blood flow.

One issue with the use of blood gases as an endpoint in a safety pharmacology study is the technical challenge to collect them. For proper evaluation of respiratory effects, the blood sample must be collected from an artery which is difficult to locate in small species such as rats and difficult to obtain safely from any species without some form of physical or chemical restraint. As a modification for access, an arterial vascular access port (VAP) can be placed surgically, prior to study activities, but then requires subsequent careful maintenance to ensure patency.

The second issue with the use of blood gases, particularly PaO_2, is that oxygen is very poorly soluble in plasma with a typical value of ~0.3 mL of oxygen per 100 mL of blood at a PaO_2 of 160 mmHg (partial pressure of O_2 in alveolar air). In contrast, the hemoglobin-bound O_2 is ~20.1 mL per 100 mL of blood at this PaO_2. Thus, the PaO_2 is only indicative of changes in the small percentage dissolved in plasma and not of the overall blood-carrying capacity.

A proposed alternative to arterial blood gases is to use the percent of oxygen saturation (SaO_2) in the blood. This is an easily measured value using light-based pulse oximetry. The limitations to the use of SaO_2 are that the total oxygen content of the blood is based on three aspects: the quantity per unit of blood of hemoglobin, the O_2-binding capacity of hemoglobin, and the percent saturation. Further, the O_2–hemoglobin binding curve is sigmoidal in shape as it reaches the 100 % saturation asymptote. Because of the flattened portion of the upper binding curve, it is possible to have the PaO_2 drop from 100 mmHg to 60 mmHg, with only a drop to 90 % for saturation values. These characteristics allow the hemostasis of oxygen supply in the presence of altered physiological parameters, and it is this characteristic that makes the use of SaO_2 of limited value for assessment of respiratory dysfunction.

The lack of sensitivity of SaO_2 for detecting blood gas changes was demonstrated in a study using healthy conscious dogs. In that study, minute ventilation was reduced by approximately 68 % following the intravenous injection of a respiratory depressant. The severe hypoventilation was associated with a 21 % increase in $PaCO_2$, a 14 % decrease in PaO_2, and no change in SaO_2 (Authier et al. 2008). Conversely, a 96 % increase in minute ventilation produced by a respiratory stimulant (albuterol) had no effect on PaO_2, $PaCO_2$, or SaO_2 (Authier et al. 2008). These findings indicate that a change in blood gases (especially SaO_2) is not a sensitive measure of drug-induced ventilator change in conscious, healthy animals as are used in standard safety pharmacology studies.

5 Conclusions

The goal of respiratory safety pharmacology studies under ICH S7A is to be able to predict the effects of test substances on pulmonary function by evaluating one or more parameters. The lungs have limited response mechanisms that are based on the nature and duration of the insult as well as the duration for compensatory responses. Like many physiological homeostatic systems, the respiratory system is overengineered, i.e., has many redundant mechanisms, thus making detection of differences due to drug administration challenging.

Core respiratory function assessment should include direct measures of both ventilation patterns and lung mechanical changes. The altered ventilatory patterns detect changes in the control of respiration, whereas altered lung mechanics can detect changes in lung function related to the ability to move air in and out of the lung. Methods for identifying mechanism, location, and/or consequences of respiratory dysfunctions are needed in order to be evaluated relative to their sensitivity and specificity for detecting a particular endpoint.

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Biophysics and Molecular Biology of Cardiac Ion Channels for the Safety Pharmacologist

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Abstract

Cardiac safety pharmacology is a continuously evolving discipline that uses the basic principles of pharmacology in a regulatory-driven process to generate data to inform risk/benefit assessment of a new chemical entity (NCE). The aim of *cardiac* safety pharmacology is to characterise the pharmacodynamic/pharmacokinetic (PK/PD) relationship of a drug's adverse effects on the heart using continuously evolving methodology. Unlike Toxicology, safety pharmacology includes within its remit a regulatory requirement to predict the risk of rare cardiotoxic (potentially lethal) events such as torsades de pointes (TdP), which is statistically associated with drug-induced changes in the QT interval of the ECG due to blockade of I_{Kr} or $K_v11.1$ current encoded by hERG. This gives safety pharmacology its unique character. The key issues for the safety pharmacology assessment of a drug on the heart are detection of an adverse effect liability, projection of the data into safety margin calculation and clinical safety monitoring. This chapter will briefly review the current cardiac safety pharmacology paradigm outlined in the ICH S7A and ICH S7B guidance documents and the non-clinical models and methods used in the evaluation of new chemical entities in order to define the integrated risk assessment for submission to regulatory authorities. An overview of how the present cardiac paradigm was developed will be discussed, explaining how it was based upon marketing authorisation withdrawal of many non-cardiovascular compounds due to unanticipated proarrhythmic effects. The role of related biomarkers (of cardiac repolarisation, e.g. prolongation of the QT interval of the ECG) will be considered. We will also provide an overview of the 'non-hERG-centric' concepts utilised in the evolving comprehensive in vitro proarrhythmia assay (CIPA) that details conduct of the proposed ion channel battery test, use of human stem cells and application of in silico models to early cardiac safety assessment. The summary of our current understanding of the triggers of TdP will include the interplay between action potential (AP) prolongation, early and delayed afterdepolarisation and substrates for re-entry arrhythmias.

Keywords

Activation • Atrial preparation • Biophysics • Calcium channel • Cardiac action potential • Channel kinetics • Comprehensive in vitro proarrhythmia assay • Delayed rectifier • Early afterdepolarisation • ECG • Hodgkin–Huxley • ICHS7A • ICHS7B • In silico modelling • Inactivation • Inward rectifier • Langendorff heart • Purkinje fibre • Safety assessment • Sodium channel • Stem cells

1 Current Cardiovascular Safety Pharmacology Studies

1.1 Ion Channel Blockade Assays

Each NCE is evaluated for potential proarrhythmic liability (particularly TdP liability) initially by evaluating potential adverse effects on cardiac ion channels in the very early stages of drug development. These 'frontloading' safety pharmacology (SP) studies are normally undertaken *before* selection of a candidate drug for development and before regulatory studies are conducted. Frontloading studies are conducted by 80 % of all companies. The safety pharmacologist is the individual primarily responsible for initiating, driving and leading the early SP assessment. Although there is increasing focus on a wide range of ion channels, all cardiac safety includes high-throughput (HT) evaluation of effects on the hERG-encoded I_{Kr} current (often abbreviated as hERG screening). The assay predominantly uses the cloned human channel expressed in either CHO or HEK cell lines (isolated myocytes are rarely used) as their primary test system. These studies are conducted at room temperature or at physiological temperatures (~37 °C). Temperature has an effect on channel kinetics; thus, physiological temperatures are recommended (Kirsch et al. 2004).

Larger companies require mandatory hERG screening prior to compound progression. Smaller companies may sell on their intellectual property (IP) rather than invest in a full SP programme. The following methodologies are used: automated patch clamp, non-automated patch clamp, ligand binding studies, and rubidium efflux studies. Selectivity screen/receptor binding profiling of the NCE is often undertaken. Other studies that are frontloaded include cardiac AP duration (APD) measurement in rabbit guinea pig and dog and haemodynamic/ECG studies in dogs or rodents (Lindgren et al. 2008).

Subsequently, either additional ion channel studies or more sophisticated voltage-clamp methods may be used in conjunction with in vitro APD studies to assess proarrhythmia liability. In vivo models may be used before selection of the candidate drug, but use is limited due to their low-throughput nature. Prior to first in human (FIH) studies in vitro APD and in vivo APD and QT interval assessments are usually undertaken to complement the early cardiac screening data, thus establishing a dataset used to inform an integrated risk assessment as described in the ICH S7A and ICH S7B guidelines (Anon. 2001, 2005). Almost no non-clinical safety work is conducted after FIH.

In summary, a large range of in vitro and in vivo cardiovascular (CV) studies are used by safety pharmacologists in order to fully characterise the safety profile of a lead candidate molecule.

1.2 QT as a Surrogate Biomarker and Studies Used to Assess Ion Channel Modulation

The cardiovascular safety core battery of tests evolved over many years due to the inherent difficulty in reaching consensus about how to safely test for risk of a rare but potentially lethal drug-induced cardiac syndrome known as TdP. Non-clinical detection of a clinically rare adverse event is difficult to undertake if the adverse event is equally rare in animals. Since this is the case for drug-induced TdP, a surrogate biomarker was needed. However, as surrogate biomarkers for rare events are difficult to validate (where validation requires the accumulation of a large database of positive and negative controls) and there is uncertainty over the extent of the risk that clinically established drugs have for generating the adverse effect in patients (no 'gold standard' or quantitatively structured template of human response to drugs), then reliable validation of the biomarker becomes an elusive (if not impossible) goal. This summarises the situation with TdP risk assessment. Today, a uniform international consensus exists regarding mandatory cardiovascular safety testing methods, which is, predictably, conservative and exhaustive: ICH guidelines S7A and S7B.

Since the 1970s, there has been a growing awareness that widening of the QT interval of the ECG is statistically associated with a risk in development of drug-induced TdP (Malik and Camm 2001; Shah 2001). This was initially recognised only for 'cardiovascular-targeted' therapeutic drugs such as prenylamine, bepridil, sotalol and quinidine (Vos 2001). However, it was then found that 'non-cardiovascular'-targeted drugs could also precipitate TdP and that this appeared to be most common among drugs that also widened QT interval (DePonti et al. 2002). In the 1990s, many well-known drugs were withdrawn from the market as a result of TdP liability, including terfenadine and astemizole (second-generation antihistamines), cisapride (a prokinetic GI agent with cholinomimetic and 5-HT_4 receptor agonistic properties), terodiline (an anticholinergic/antispasmodic drug effective in the treatment of urinary incontinence) and grepafloxacin (a fluoroquinolone antibiotic) (DePonti et al. 2002). This precipitated the collaboration between pharmaceutical companies and regulatory authorities that led to the construction of the guidance documents we now know as ICH S7A and S7B.

When the S7A guidance was published, selecting a method for evaluating TdP liability was an issue that was not addressed. Ventricular repolarisation delay is generally regarded as the best, albeit still poor, biomarker for TdP risk. A degree of consensus with respect to approach has been achieved, albeit through compromise regarding what actually constitutes a threshold transgression (in terms of magnitude of threshold dose and magnitude of threshold effect) for different putative TdP biomarkers such as prolongation of the QT interval and blockade of the cardiac potassium current I_{Kr} (Cavero and Crumb 2005). Despite the years since implementation of the SP guidance documents, there is still no agreement regarding the identification of a single best preclinical animal model for detection of TdP liability, and instead the compromise has been adoption of an integrated risk assessment strategy (Pugsley et al. 2008).

Cardiac SP in vivo methods primarily use conscious telemetered animals to assess the effects of the test item on the cardiovascular system. Variables that are recorded in the dog include blood pressure, heart rate and ECG (see Authier et al. 2015). Complementary studies, usually non-GLP in nature, include a number of in vitro assays that have been well characterised with utility in the safety profiling of an NCE. These assays include assessment of drug effects in the isolated guinea pig right atrium preparation, rabbit Purkinje fibre preparation, the isolated Langendorff heart and the isolated wedge preparation. Each assay will be briefly discussed.

1.2.1 The Isolated Atrial Preparation

The types of isolated tissues that can be used to assess drug effects on the heart are extensive and remain a cornerstone of not only the physiological and pharmacological evaluation of natural and synthetic drugs but also proved a means by which to characterise cardiac safety. The cardiovascular system is a rich source of tissue for in vitro studies. Some of the earliest assessments of a drug effect (i.e. not simply a CEREP non-functional binding study) can be made in isolated cardiac tissues, such as the isolated atrium. Such preparations can involve strips of atrial tissue (such as from the rabbit) or the entire atria (left and right) but usually just involve the single right atrium. Intact preparations are particularly useful in assessing the nature of a drug on cardiac function in terms of changes to beating rate, force of contraction, membrane potential as well as determining biochemical activity. Recently, Goineau et al. (2012) compared the sensitivity of three SP models that could be used in the assessment of drug-induced effects on cardiac conduction. They observed that rabbit atria were more sensitive at detecting lidocaine-induced cardiac slowing than rabbit Purkinje fibres or guinea pig atria.

Anatomically, in adult mammals, the right atrium is primarily used as it is larger than the left atrium and contains the anatomical structures necessary for cardiac conduction and the coronary circulation. Additionally, the right atrium spontaneously beats at a rate dominated by the sinus node and because atrial cells contain numerous G-protein-coupled receptors (GPCR) and ion channels provide a 'multiple target' testing assay. However, delineation of the mechanism of action for the drug effect is not easy to establish and would require follow-up studies. The strength of the assay is that it can be used as an early safety screen to direct medicinal chemistry synthesis of compounds that lack untoward cardiac effects. Moreover, drugs can be added directly to an organ bath, and there is no requirement for technically difficult aortic cannulation (required for the Langendorff model—see later) and so the technique maybe applies with ease to mouse models, in which gene modification studies are most conveniently undertaken. This allows for heritable risk to be factored into assessment.

1.2.2 The Isolated Purkinje Fibre

Cardiac Purkinje fibres are subendocardially located within the heart along the inner ventricular wall and are responsible for the spread of myocardial electrical activity that results in the generation of the QRS complex of the ECG. These fibres, first described by the Czech physiologist and anatomist Johannes (Jan) Evangeline

Purkinje (Purkyně) in 1839, are comprised of specialised cardiomyocytes with a distinctive ion channel profile that effect rapid conduction of an electrical impulse (~4.0 m/s) from the bundle branches through the heart to the working myocardium the point of termination of the cardiac conduction system (Sedmera and Gourdie 2014). It is the fast conduction in the Purkinje fibre that establishes the cardiac conduction synchrony of the ventricles from apex to base that maintains a consistent heart rhythm. However, it is this same property of these fibres that can make the heart susceptible to, or perpetuate, the development of re-entrant arrhythmias and abnormal automaticity (Boyden et al. 2010). Purkinje fibres are large cells with limited contractile elements and fewer mitochondria and conduction does not respond to changes in autonomic tone compared with cells of the working myocardium (Boyden et al. 2010). In SP the assay may consist of Purkinje fibres isolated from the rabbit, guinea pig or dog and usually involves determining the concentration–response profile and rate dependence of the NCE on AP parameters. These standard parameters include the AP amplitude (APA), maximum rate of ventricular depolarisation (V_{max}) and APDat 60 % and 90 % repolarisation (APD_{60} and APD_{90}). Additionally the magnitude of the effects of the NCE should be routinely compared to vehicle and usually a positive control (such as sotalol) conducted in parallel within the study.

SP studies have shown that isolated rabbit Purkinje fibres, when used in the assay, are highly sensitive to drugs with a greater propensity to prolong the APD and elicit early afterdepolarisations (EAD) compared to fibres from guinea pig, dog or swine (Lu et al. 2000, 2001). Thus, it appears that it is a highly specific and sensitive model to investigate drug-induced TdP liability. Lu et al. (2008) have also shown that there are marked differences in tissue response to drugs when isolated rabbit Purkinje fibres are used in the assay compared to either papillary muscle with ventricular trabeculae. Over the years, the assay has been well validated through the testing of many reference compounds (see Champeroux et al. 2005; Hanson et al. 2006; Aubert et al. 2006; Puddu et al. 2011) for use in the early de-risking stages of development (as a non-GLP assay) or as a component of the GLP-compliant in vitro core battery testing scheme. It has been identified as an important non-clinical assay in drug safety assessment by global regulatory authorities (Corrias et al. 2011).

1.2.3 The Langendorff Isolated Heart

The perfused isolated heart has many advantages in the study of the actions of an NCE on the mechanical (i.e. inotropic and lusitropic effects) and electrical properties of the heart (Bell et al. 2011; Clements-Jewery and Curtis 2014). The isolated heart was first described by Langendorff in 1895 as a simple preparation with which to study the activities of drugs. Briefly, hearts are perfused through the aorta with an oxygenated physiological buffer solution using a methodology essentially unchanged since originally described. The isolated heart is free of extrinsic nerves (but does contain a rich intrinsic innervation of ganglionic plexuses; see Brack 2014) and circulating systemic hormonal factors as well as variation in haemodynamics that may alter drug activities. A constant perfusion pressure closes

the aortic valve facilitating coronary artery blood vessel perfusion. The highly vascularised cardiac muscle ensures that exposure to drugs carried within the buffer rapidly gains access to all myocytes providing a sensitive measure of drug effects on contractility (mechanics), coronary flow and the electrocardiogram (ECG). This model is used extensively in SP studies (Lawrence et al. 2006; Guo et al. 2009) and is widely recognised as a surrogate for the study of human cardiac function and considered a conduit between in vitro cellular studies (hERG) and in vivo (conscious dog) screening methods. The nature of the preparation allows for hearts from a wide range of non-clinical species to be studied and may better predict effects observed in vivo than single cell studies since the syncytium is intact (Clements-Jewery and Curtis 2014). The rabbit and guinea pig hearts are used extensively in drug safety assessment since they show comparable electrophysiological responses to that observed in human hearts when similar drugs are investigated (Hondeghem and Hoffmann 2003; Hamlin et al. 2004). Thus, the Langendorff isolated heart provides a means by which to assess the safety profile of NCEs contiguously on electrical, mechanical and biochemical properties of the heart (Clements-Jewery and Curtis 2014; Curtis 1998).

1.2.4 The Isolated Coronary-Perfused Wedge Preparation

The isolated coronary-perfused wedge preparation is a commonly used non-clinical model in SP (Lee et al. 2010) that was developed by Yan and Antzelevitch (1996). The preparation is highly sensitive to detect effects of drugs while remaining selective enough to differentiate between drugs with known TdP liability drugs and non-arrhythmic drugs (Wang et al. 2008). Because of the nature of the preparation, the NCE can be continuously infused into the perfusion medium at increasing concentrations and a number of superimposed physiological alterations such as hypokalaemia, bradycardia or tachycardia (albeit limited in duration to ~60 s in order to avoid myocardial ischaemia) can be assessed. This method makes use of the marked regional differences in electrophysiology across the ventricle (intramural dispersion) that is characteristic of the preparation. Drug effects are evaluated on three distinct tissues within the ventricle: the epicardium, mid-myocardium (M-cells) and endocardium (Yan et al. 1998). The M-cells are the last to complete repolarisation and this is thought to be due to the presence of a smaller, slowly activating, delayed rectifier current (IKs), a larger late depolarising sodium current (or late INa) and a larger electrogenic sodium–calcium exchange current (Yan and Antzelevitch 1998; Antzelevitch and Shimizu 2002). The model uses electrodes that are placed at the level of each tissue region, which then record the three transmembrane potentials simultaneously, with an accompanying ECG for reference. In addition to the standard ECG measures, transmural dispersion of repolarisation (TDR) can be evaluated as the ratio of the interval between the peak and end of the T-wave and the QT interval [(Tp-Te)/QT] (Liu et al. 2006). The model also detects Phase 2 EADs and EAD-mediated R-on-T extrasystoles and an arrhythmia score can be applied for a semi-quantitative estimate of TdP potential.

The wedge preparation has been criticised on the grounds that transmural dispersion of the magnitude expressed is not found in an intact heart in which cell coupling largely precludes it (Curtis et al. 2013). However, this is not relevant if the model accurately predicts TdP liability with precision and accuracy. Indeed clinical relevance in the context of SP must be and need only be measured in terms of predictivity, and not the degree of identity between screen readout and human physiology.

While the isolated coronary artery-perfused ventricular wedge preparations have been shown to have a high specificity and selectivity for human torsadogens (Antzelevitch 2004; Liu et al. 2006), perceived limitations include the in vitro setting, the requirement for technical expertise and familiarity and the consequence that the model is not fit for high-throughput SP.

1.3 The Comprehensive In Vitro Proarrhythmia Assay

The clinical thorough QT (TQT) study (as outlined in the ICH E14 guidance) in conjunction with the series of non-clinical studies focused on assessing hERG current block (as outlined in the ICH S7B guidance) has effectively reduced the risk of developing (and subsequently approving) new drugs with the potential for precipitating torsades de pointes (TdP) cardiac arrhythmias. However, development and current conduct of this paradigm have come at a high cost to both the pharmaceutical industry in terms of the actual added cost to develop new drugs (i.e. conduct of the TQT study is ~$4M/study) and society in terms of the loss to patients of potentially novel, effective drugs that have a hERG liability (or potential to prolong the QT interval) but with a low potential for proarrhythmia.

A novel cardiovascular risk assessment paradigm is being developed that would help to obviate conduct of the clinical TQT study (Darpo et al. 2014). The proposed Comprehensive In vitro Proarrhythmia Assay (CIPA) paradigm aims to modernise and provision current non-clinical, 'hERG-centric' cardiac safety screening efforts (Sager et al. 2014). A number of challenges and opportunities exist that are associated with adoption of a new approach in the evaluation of the arrhythmia potential of new drugs using novel in vitro (human stem cells) and in silico (cardiac AP modelling) methods and their application to clinically relevant arrhythmias such as TdP.

1.3.1 Stem Cells and CIPA

Current alternative screening models and methods under consideration by the SP and regulatory communities for the CIPA initiative include stem cells in which hERG (I_{Kr}) as well as other cardiac ion channels such as sodium (SCN5A), calcium (Cav1.2) and some potassium channels (I_{K1} and I_{Ks}) can be assessed in totality (Pugsley et al. 2014). Rather than examining human ion channel isoforms heterogeneously expressed in cell lines (such as CHO or HEK) as is current practice in drug safety or, on the rare occasion, actually using isolated human cardiac myocytes, the community is investigating applicability of human-induced

pluripotent stem cells (Vidarsson et al. 2010; Peng et al. 2010). The undifferentiated human stem cell of embryonic origin (hESC) and induced pluripotent stem cell (iPSCs) of somatic origin (the latter with the potential advantage that they can be obtained from diseased patients, e.g. congenital LQTS) continue to be evaluated for all aspects of their cardiac electrophysiological potential (Tanaka et al. 2009). The use of stems cells, despite being an evolving technology, may have implications in SP testing related to the CIPA initiative. It has been suggested that the use of human stem cells would be desired in order to assess drug effects on cardiac ion channels, rather than using heterologous expression systems. These cells would be an alternative model to the use of isolated human cardiac myocytes which have been plagued with numerous limitations for many years. Thus, primary induced pluripotent cardiac stem cell (iPSC-CM) of somatic origin would be preferred for use compared to undifferentiated human stem cells of embryonic origin (hESC). Embryonic stem cells clearly have limitations that undermine their place in non-clinical research. However, primary iPSC-CM are being evaluated for cardiac electrophysiological properties and potential for use as a drug screening assay (Tanaka et al. 2009; Peng et al. 2010; Vidarsson et al. 2010; Gibson et al. 2014). Human iPSC-CM have the additional potential advantage that they can be obtained from diseased patients, e.g. with congenital LQTS. Intracellular recordings from individual cells (Peng et al. 2010) and multi-electrode arrays (MEA) enable measurement of sodium (SCN5A), calcium (Cav1.2) and potassium (I_{Kr} or hERG) current.

Implementation in CIPA will provide the impetus for conduct of ion channel blockade validation studies using stem cell technologies in order to establish a case for their use as an early drug screening assay or cardiovascular SP study model (with the hope they may be used routinely in the future as part of the core battery cardiovascular studies). However, not until phenotypic consistency, ion channel expression and electrophysiology profiles and responses to control drugs are established in terms of sensitivity, specificity and predictive utility are shown to be favourable versus current, established non-clinical models will stem cells transcend their status as secondary or tertiary cardiovascular assays. High-throughput screening (HTS) methods involving the use of stem cells create additional experimental concerns compared to those involving standardised CHO or HEK cell lines. A major constraint, in the foreseeable future, for the use of stem cells is their availability for HTS applications because of the number of cells required in automated chip-based patch-clamp systems. It should be remembered that a cell does not provide a complete physiological response.

1.3.2 In Silico Methods with Application to SP

For many years, there has been an ongoing effort to mathematically describe the cardiac AP (see below). Primarily driven by academic research groups, there has been a recent shift involving the integration of these researchers with those in industry in order to apply in silico methods to the CIPA initiative. For example, Mirams et al. (2014) characterised ion channel blockade for the series of ion channels that are responsible for genesis of the cardiac AP and proposed CIPA

candidates. These include hERG, the L-type calcium channel, the inward sodium channel, the KCNQ1/MinK or $K_v7.1$ (K_vLQT1) and transient outward K current). Mirams et al. (2014) used two standard high-throughput screening (HTS) assay systems to generate the data employed in the in silico models to simulate drug-mediated effects on human ventricular APs. Several mathematical simulation models were tested including the ten Tusscher and Panfilov (2006), Grandi et al. (2010) and O'Hara et al. (2011) models. Data derived from these in silico concentration–response simulations were compared to clinical TQT results for the 34 compounds tested. At the study conditions used (1 Hz pacing and channel block determined at steady state) simulations tended to underestimate the QT prolongation observed in the clinic. Note that there are some differences, albeit subtle, between the numerous in silico methods utilised in AP simulation.

ten Tusscher and Panfilov (2006) developed a model of the cardiac ventricular AP based upon the measurement of human APD restitution, extensive description of intracellular calcium dynamics and importance of sodium channel recovery dynamics. The latter is important in defining the occurrence of electrical instability. Grandi et al. (2010) developed a mathematical model of the ventricular AP that characterised calcium handling as well as ionic currents measured in human cells. In development of their model they simulated basic excitation–contraction coupling phenomena and applied repolarising potassium current densities derived from a previously established rabbit myocyte model. However, unlike other models this one includes subsarcolemmal compartments where ion channels are programmed to 'sense' higher levels of calcium compared to the cytosol. In addition, transmural gradients for Ca handling proteins and the Na pump were simulated and both the rapid and slow inactivating components of the transient outward K current (I_{to}) were simulated to differentiate between localisation either endocardially or epicardially. O'Hara et al. (2011) developed a human ventricular cardiac AP model based upon data measured from over 100 undiseased human hearts. Components of the model were evaluated over the human range of physiological frequencies and include calcium versus voltage-dependent inactivation of L-type calcium current (I_{CaL}) and kinetics for the transient outward, rapid delayed rectifier (I_{Kr}) and inward rectifier (I_{K1}) potassium currents along with the Na^+/Ca^{2+} exchange pump (I_{NaCa}). The authors also examined model response to rate dependence and restitution of cardiac AP duration (APD). Note that this model is referred to as the O'Hara-Rudy Dynamic (ORd) model.

The results derived from this ongoing research are important to development of the CIPA initiative currently ongoing between the pharmaceutical industry, contract research organisations and regulatory authorities (FDA) (Sager et al. 2014). The establishment of datasets of clinically used compounds should provide much needed information to the entire scientific community regarding adoption and applicability of in silico simulations in safety hazard identification as well as the nature of the data derived from HTS (vs. patch clamp) methods subsequently used in simulation methods.

Thus, application of in silico methods, with noted limitations, may have use in studies preceding frontloading SP cardiovascular studies provided that the

respective participants (companies or CRO laboratories) have the capability (i.e. informational technology computing capability, technical understanding of the mathematical models used in the model construction, etc.) to incorporate such assays into discovery screening procedures. Introduction could increase throughput and limit development of chemical scaffolds with potential cardiac liability.

The anticipated outcome from this change in paradigm is the development of a non-clinical, standardised in vitro assay that determines the effects of drugs on the major cardiac ion channels and provide an assessment of the potential to precipitate clinical proarrhythmia, obviate conduct of the clinical TQT study and facilitate more efficient drug discovery efforts. In order to understand the fundamentals of this novel paradigm, it is important to review fundamental cardiac electrophysiology including basic ion channel biophysics as well as review some fundamental cardiac arrhythmia mechanisms to achieve this alternate approach.

2 Myocardial Cell Types and Myocyte Coupling in the Heart

The safety pharmacologist, when conducting cardiac studies, should know that within the myocardium cardiac cells (myocytes) differ both in their morphology and electrophysiological properties. This distinction must be clearly appreciated since this cellular disparity exists between atrial and ventricular myocytes as well as transmurally within ventricular muscle. At least five distinct types of cell can be distinguished based upon anatomical, morphological and electrophysiological properties. These cell types are found in the sino-atrial node (SAN) tissue, atrial muscle, atrio-ventricular node (AVN) tissue, His-Purkinje fibres and ventricular muscle. In general these cells, usually quadrangular in shape (50–100 μm in length by 10–20 μm in width), though bounded in the usual way by their cell membranes (the sarcolemma) and separated from their neighbours by extracellular fluid, are also anchored to adjacent cells, mainly end to end, by adhesive complexes called intercalated discs.

The intercalated disc is composed of three structurally distinct regions: (a) the *macula adherens or desmosome* which is a complex where the central lamella appears to receive filamentous projections from the sarcolemma providing integrity to muscle during contraction, (b) the *fascia adherentes* or 'intermediate junction' where actin myofibrils terminate, and (c) the *nexus* or gap junction which provides a low impedance pathway permitting the conduction of APs from cell to cell.

These unique sites of cellular communication sanction the propagation of electrical impulses, and hence contraction, from their origin in SAN pacemaker cells to the ventricles. Thus, the heart functions as an electrical and mechanical syncytium in which the anatomical arrangement and diverse electrical properties of cells serve to produce the sequential cell activation that underlies the coordinated function of the heart.

3 Bioelectrical Properties in the Myocardium

The electrical properties of cardiac myocytes are best introduced and described in terms of circuit elements. These concepts are of relevance to understanding of drug–ion channel interactions and hence safety characterisation of a new chemical entity (NCE) or biological drug. Important components of these idealised circuits include conductors, capacitors and voltage generators (or batteries) (Hubbard et al. 1969). An understanding of how the cardiac AP (AP) is generated and propagated depends upon both knowledge as to how these elements are arranged in cells and their relation to electric current. Electrical *current* is simply defined as a flow of ions (of either positive or negative charge). By convention, current flows from a positive to a negative direction. Thus, cations (positive ions) such as K^+ and Na^+ flow in the same direction as current. The *potential* (or *voltage*) gradient is the force that causes charged particles to move. In a *conductor*, Ohm's law is followed such that $I = GV$ or $V = IR$ where R, resistance, is the inverse of conductance (G) and V (voltage) is the potential difference between the points between which the current, (I), flows. Thus, in a conductor (such as a myocardial cell membrane), there can be no voltage gradient unless there is current flow and no current flow unless there is a voltage gradient. Ionic (or salt) solutions are Ohmic. It is thought that these charged particles (ions) move in an electric field rapidly and thus reach a limiting velocity; the point at which 'frictional' forces balance the electrical force and current becomes proportional to the voltage gradient. The heart (not unlike the whole human body) can be thought of as a 'volume conductor', which can be simulated by a three-dimensional network of linked resistors. This simulation of the heart can result because myocardial cell membranes act as capacitors. While anisotropicity, the non-uniform conductivity that depends upon current direction, is characteristic of many tissues (muscle in particular), it is especially important to the heart as it ensures proper uniform excitation of cardiac muscle and contraction (Pugsley and Quastel 1998).

Capacitance results whenever two conductors are separated by an insulator. In this case, a potential difference can be maintained indefinitely between the two conductors. Then, by definition, $Q = CE$ and $I = \mathrm{d}Q/\mathrm{d}t = C\mathrm{d}E/\mathrm{d}t$ where Q is charge and capacitance is C. When the voltage across a capacitor is altered charged particles move to or from both sides; this in effect results in a current 'through' the capacitor that is proportional to the rate of change of the voltage. Conversely, the voltage across a capacitor cannot change instantaneously; rather the charging or discharging of the capacitor is time dependent.

3.1 The Nernst Equation for Membrane Potential

Electrophysiological studies show that most cells have an average resting membrane potential of −80 mV. This value has been attributed to unequal concentrations across the membrane of ions to which the membrane is permeable. The myocardial cell can be thought of as being composed of two compartments

corresponding to the inside and outside of the cell. These compartments are separated by a membrane that is permeable to only one ion, K^+ for example. Thus, inside the cell are K^+ ions and the charge on these ions is balanced predominantly by large negatively charged (anionic) protein molecules which cannot penetrate through pores in the membrane. Outside the cell there is a relatively low concentration of K^+ ions and other additional ions that cannot pass through the membrane. In order for this model to be effective, it is assumed that there is no potential across the membrane. More intracellular K^+ ions, by thermal movement, leave the cell through the membrane pores than enter. Since the K^+ ions move outward from the cell and are not accompanied by anions, the result is a net negative charge in the cell. This negative charge then opposes the exit and promotes the entry of K^+. It can be calculated that this exactly balances the tendency of K^+ ions to flow from high to low concentration when the transmembrane potential is that given by the Nernst equation (Pugsley and Quastel 1998). Thus for any ion, J, with concentrations $[J]_o$ outside and $[J]_i$ inside, and valence z_J this equation states that $E_J = RT/z_JF \cdot \ln([J]_o/[J]_i)$ and for an ion such as K^+ this equation can be written as $E_K = RT/F \cdot \ln([K^+]_o/[K^+]_i)$. In these equations, R is the universal gas constant, T is the absolute temperature and F is the Faraday constant (96,500 C/mol). E_K is termed the '*equilibrium potential*' or '*reversal potential*" for K^+ ions and it is the transmembrane voltage at which the net flow of K^+ ions through the membrane is zero. In this hypothetical cell model, the concentration difference across the membrane results in a stable situation where transmembrane potential is maintained at E_K and $[K^+]_i$ remains constant. Note that the myocardial cell membrane is permeable to a number of anions and cations, so each of the resulting derivations of the above equation must consider the valence of the ion in question. Thus for a membrane that is permeable to any of these ions, modifications can be made to the equation. For Na^+ ions, the equation can be written as $E_{Na} = RT/F \cdot \ln([Na^+]_o/[Na^+]_i)$ while for Ca^{2+} ions $E_{Ca} = RT/2F \cdot \ln([Ca^{2+}]_o/[Ca^{2+}]_i)$ and for Cl^- ions $E_{Cl} = -RT/F \cdot \ln([Cl^-]_o/[Cl^-]_i)$ or $E_{Cl} = RT/F \cdot \ln([Cl^-]_i/[Cl^-]_o)$.

For a cell membrane that has channels allowing permeation of more than one type of ion, the resting membrane potential will evidently result from a compromise between the equilibrium potentials for each of the various contributing ions. Two approaches can be used to calculate cell potentials. The first method calculates the theoretical current carried by each ion at various voltages. The Goldman–Hodgkin–Katz (GHK) current equation (Hille 1992) was developed based on the assumption that the electrical field in the membrane is constant. It states that for any ion J that $I_J = P_J z_J F \cdot v_J \cdot ([J]_i \exp(v_J) - [J]_o)/(\exp(v_J) - 1)$ where $v_J = V/(RT/z_JF)$. In this equation, P_J is the membrane permeability to the J ion and V is the potential across the membrane. As a result, in this equation current is non-linearly related to voltage, i.e. the membrane becomes a non-Ohmic conductor, unless the ionic concentrations on the two sides of the membrane are equal. The GHK voltage equation (Hille 1992) can be easily acquired by determining the voltage at which the sum of all currents is zero. For a membrane that is permeable only to K^+, Na^+ and Cl^- and for which no other source of membrane current exists, the equilibrium or resting membrane potential, V_r, is $V_r = (RT/F) \cdot \ln\{(P_K[K^+]_o + P_{Na}[Na^+]_o + P_{Cl}[Cl^-]_i)/$

$(P_K[K^+]_i + P_{Na}[Na^+]_i + P_{Cl}[Cl^-]_o)\}$. In fact, this equation can be correctly applied when the electrical field in the membrane is not uniform.

Physiologically, the Cl^- ion terms in these equations can often be ignored. With a high external Na^+ and high internal K^+, the resultant V_r (whatever its value may happen to be between E_K and E_{Na}) cannot be sufficiently negative to prevent the continuous egress of K^+ ions and entry of Na^+ ions. Therefore, stability of the cell becomes paramount and requires an active (and perpetual energy consuming) ionic transport mechanism. The 'sodium/potassium pump exchanger' then continuously extrudes Na^+ ions and imports K^+ ions.

The second approach in the derivation of the resting membrane potential of a cell considers the equivalent circuit of the membrane. With a separation between the inside and outside of the cell, there develops a capacitance and voltage potential, with an internal resistance (or conductance) that corresponds to each of the relevant permeant ions. Simple mathematical equations can be developed for each ion such that for K^+ ions $I_K = g_K\ (V - E_K)$, for Na^+ ions $I_{Na} = g_{Na}\ (V - E_{Na})$, for Cl^- ions $I_{Cl} = g_{Cl}\ (V - E_{Cl})$ and for Ca^{2+} ions $I_{Ca} = g_{Ca}\ (V - E_{Ca})$. As with the GHK equations for the 'resting' state of membrane currents, the sum of all currents is zero and the equation becomes $V_r = (g_K E_K + g_{Na} E_{Na} + g_{Cl} E_{Cl} + g_{Ca} E_{Ca})/(g_K + g_{Na} + g_{Cl} + g_{Ca})$. With this model, there is a continuous inward Na^+ and Ca^{2+} current that is balanced by the sum of many outward K^+ currents (see below for details on K^+ current subtypes involved). However, unlike the GHK current equation, this approach does not account for the fact that, in general, conductance should be non-linear, i.e. it should depend upon voltage, since each ion exists in unequal concentrations on either side of the membrane. However, this equivalent circuit model can be applied if it is assumed that conductance is nearly a constant when associated with small changes in membrane potential and that, in myocytes, changes in the potential of the cardiac cell membrane are associated with *large* changes in conductance.

In an attempt to refine the above formulation, it was thought that perhaps it might be more accurate to describe the components of the equivalent circuit (i.e. the combination of battery and internal resistance) for each ion *channel* rather than to a particular ion. As well, to impart relevance to the system to accurately model the cardiac myocyte (as has been done by Noble & Mirams, O'Hara & Rudy and ten Tusscher & Panfilov amongst others), this approach should include as many different types of physiological ion channels as possible. Each voltage (or equilibrium potential) is represented not by the Nernst equation for a particular ion but rather by the GHK voltage equation using both ion concentrations and permeability ratio(s). This approach was formally described for currents in the mammalian ventricular cell by Luo and Rudy (1991) who used Na^+ and Ca^{2+} currents, three distinct K^+ currents as well as a 'background' K^+ current with an equilibrium potential between E_K and E_{Na}.

The equivalent circuit model of the membrane is best visualised schematically where capacitance represents the insulating myocardial lipid bilayer and each conductor–voltage (battery) combination represents a type of ionic channel.

Similarly for the more mathematically inclined, this may be a simple mathematical model such that the sum of all currents ($C\mathrm{d}V/\mathrm{d}t$) must be zero.

The greatest physiological application for the equivalent circuit model is where myocardial cells are represented as a 'ladder' network. In the case of the heart, myocytes are represented as being connected end to end (which they are within the heart) with an internal resistance (R_i) between two cells. Within the context of the heart, it must be realised that myocytes are connected to adjacent cells and therefore the network most accurately reflects the physiological scenario when considered in two or even three dimensions.

4 Voltage-Gated Ion Channels and Genesis of Cardiac APs

Our current understanding of electrical activity of cardiac muscle is based upon the observation that transmembrane potentials result from the coordinated integration of the opening and closing of many different ion channels. The corresponding changes in ionic permeability and conductance result in the flow of current, genesis of an AP and contraction of heart muscle.

Development of the theory of AP genesis and propagation in nerve and cardiac muscle resulted from the work of Hodgkin and Huxley (1952). APs in excitable cells result from the presence of voltage-gated ion channels that open and close depending upon the voltage across the membrane. Depolarisation of the resting membrane potential causes the opening of voltage-gated Na^+ channels. This increase in Na^+ permeability (or conductance, g_{Na} or G_{Na}) results in the development of an inward current which enhances depolarisation and increases g_{Na}. This, in turn, further increases the inward current and g_{Na} until a maximum is attained. If this were the case, the membrane potential would be permanently shifted to E_{Na} in response to *any* depolarisation. Several factors prevent this event from transpiring. These include the fact that resting conductance allows for only a small increase in g_{Na} and inward current (which does not adversely affect the transmembrane potential), voltage-gated Na^+ channels rapidly inactivate (within several milliseconds after depolarisation) and depolarisation marks the opening of voltage-gated K^+ channels which shifts the membrane potential back to E_K. In contrast to nerves, the situation in the heart is complicated by the presence of voltage-gated Ca^{2+} channels. These channels mediate the 'slow inward current' (I_{si}) that is predominantly responsible for the plateau phase of the AP (see details below). In some tissues, such as the sino-atrial and atrio-ventricular nodes, voltage-gated Ca^{2+} channels predominate and provide a basis for the AP.

4.1 Hodgkin–Huxley Equations

It was from studies and conclusions based upon the classical work of Hodgkin and Huxley (1952) that provides us with a basis for a mathematical description of voltage-dependent ion channel conductance. Their experimental strategy was

essentially to record electrical activity inside a giant squid axon cell using a 'voltage clamp'. Experimental evidence however did not support the predicted change in current amplitude. The theoretical model predicts that if conductance were constant, then steady current responses should result, and this was not observed. Rather, experimental currents were not maintained in a steady state. The initial inward Na^+ current reached a maximum quickly but with time subsequently declined. An outward K^+ current that slowly reached a maximum immediately followed decline in this current. Recordings of these currents at the given voltages that generated them allowed for a calculation of the conductance of each ion channel type involved in electrical activity in the axon. The fundamental feature observed for K^+ ion channels was that with depolarisation the sigmoidal rising phase of the conductance curve for this ion could be mathematically defined as an exponential rise to a maximum. Potassium channel conductance could be expressed as $g_K = G_K \cdot n^4$ where G_K is the maximal possible g_K and n is a parameter (usually less than 1.0) that changes according to the equation $dn/dt = \alpha_n(1 - n) - \beta_n n$ or $dn/dt + (\alpha_n + \beta_n)n = \alpha_n$ with both α_n and β_n being a constant at any given transmembrane potential, V. Experimentally a change to a new constant voltage would allow for a new set of values for α_n and β_n. These values are such that when the difference for n is determined for its final value, the path it follows is an exponential decrease in time course where the time constant, τ_n, equals $1/(\alpha_n + \beta_n)$.

This mathematical formalisation for the experimentally obtained values allowed α_n and β_n to be obtained for any given membrane potential. Thus, since n_f is given by the 4th root of the final g_K divided by maximum possible g_K, the resulting equations determined by Hodgkin and Huxley resembled those obtained by the GHK current equation. It was theorised that the set of obtained equations for K^+ correspond to the gating or movement of 4 'n' particles within the membrane and that the 'on' rate value for movement could be described by α_n and the 'off' rate value by β_n (Hodgkin and Huxley 1952).

By using a similar recording approach and mathematical analysis profile for the rapidly activating and inactivating Na^+ current, it was found that g_{Na} could be described by the equation $g_{Na} = G_{Na}\, m^3\, h$. In this equation, the 'm' term describes the activation properties of the channel while the 'h' term describes the 'inactivation gate' the actions of which are governed by the time-independent parameters α_m and β_m and α_h and β_h, respectively. Mathematically (and experimentally) 'm' increased while 'h' decreased with membrane depolarisation. From their work, Hodgkin and Huxley showed that they could mathematically reconstruct the time course (and magnitude) of the squid axon AP.

4.2 Genesis of the Cardiac AP

The cardiac AP, illustrated in Fig. 1 for a ventricular myocyte, is conventionally composed of four phases. A rapid upstroke (phase 0) is followed by a brief peak (phase 1) followed by a sustained plateau (phase 2). A rapid repolarisation (phase 3) begins after several hundred milliseconds have elapsed and this is followed by

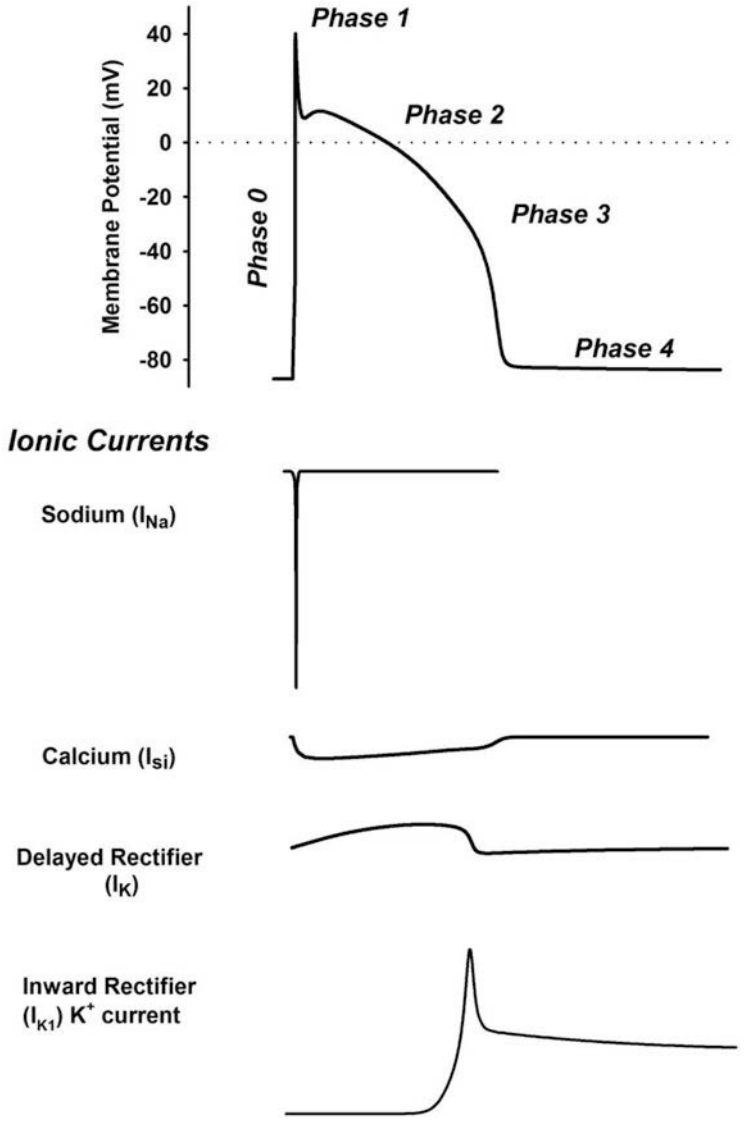

Fig. 1 The cardiac action potential (AP) (*upper panel*) conventionally consists of several phases (0–4) with a duration of approximately 300 ms. Phase 0 corresponds to membrane depolarisation (Na^+ influx), while phase 1 shows the early rapid repolarisation of the membrane. Phase 2 is the plateau of the AP (due to a reduction in Na^+ influx and increase in Ca^{2+} influx), while phase 3 shows membrane repolarisation (resulting from the coordinated opening and closing of many different K^+ channels). Phase 4 corresponds to the resting membrane potential. The *lower panels* depict the currents produced by the movement of several different ions across the membrane. By convention, both the inward Na^+ (I_{Na}) and Ca^{2+} (I_{Ca}) ionic currents are shown downward. Several of the outward K^+ ionic currents responsible for repolarisation are shown: the delayed rectifier (I_K) and the inward rectifier (I_{K1}). *Note that current amplitudes are not shown to scale*

phase 4 that persists until the next rapid upstroke event. Thus, the shape of the AP is governed by ionic current flux via gated channels in the membrane for sodium, calcium and potassium (Fig. 1). As well, membrane pumps and exchangers such as for Na/K-ATPase and Na/Ca are involved. The properties of the AP change moderately amongst tissue types. In pacemaker cells of the nodal tissues (and to a lesser degree in atrial cells and Purkinje fibres), phase 4 is characterised by a slow steady depolarisation from the resting membrane potential (V_m) that leads to a 'threshold' potential (TP). When this potential is met, a rapid upstroke (phase 0) results and a nodal AP develops that is composed of similar 'phases' as in ventricular or other cardiac cells. While there may be some differences in regard to phase 4 development in various cardiac tissue, the fundamentals of AP generation remain essentially unchanged. The pacemaker current shapes the periodicity of oscillations in the heart since this current is activated by the hyperpolarised cell membrane at the conclusion of the AP.

The ventricular AP has been mathematically modelled by several groups of researchers (Hille 1992; DiFrancesco and Noble 1985). In each model, the genesis of the AP depends upon an accurate account of the biophysical and physiological properties of the component ionic currents. These properties are then integrated into a series of uniform equations (which are based upon those of Hodgkin and Huxley) and the AP model developed.

The inward, fast voltage-gated Na^+ channel (g_{Na}) is responsible for producing phase 0 and the rapid upstroke of the cardiac AP. It is best approximated by the equation $g_{Na} = G_{Na}\, m^3 \cdot h \cdot j$, where the j represents a slow inactivation gate component of the current which has its own voltage-dependent α and β rate constants.

While Na^+ channels rapidly inactivate as the membrane potential (V_m) approaches the equilibrium potential (0 mV), a second voltage-gated ion channel is activated that is carried by Ca^{2+} ions. Calcium channels carry I_{si} that is responsible for the plateau phase of the AP (Fig. 1). These channels activate rapidly at approximately −40 to −20 mV and inactivate slowly. Calcium current can be approximated by the equation $g_{Ca} = G_{Ca} \cdot d \cdot f$ where voltage-dependent rate constants (α and β) are determined for d and f. Many Ca^{2+} channel subtypes occur in the body. In the heart, at least 2 isoforms can be found: the 'L' and 'T' types (see below for details).

Within a short period of time (≈150 ms), cardiac Ca^{2+} channels begin to inactivate and K^+ channels begin to activate. Repolarisation becomes relatively rapid when the total outward K^+ current becomes appreciably greater than inward Ca^{2+} current. A large number of voltage- and non-voltage-gated K^+ currents are involved in repolarisation of the cardiac AP. The voltage-gated K^+ currents include the transient outward K^+ current (I_{to}), one of the earliest channels to open (during phase 1) to begin repolarisation, the outward or delayed rectifier current (I_K) which opens and the end of phase 2 and is the main K^+ current responsible for ventricular repolarisation and the inward rectifier K^+ current (I_{K1}) which, unlike other K^+ currents, closes during depolarisation and is responsible for maintenance of the resting membrane potential (see the lower panel of Fig. 1 for details).

An integration of the equations discussed above can accurately predict the shape of the AP. These equations can not only predict the shape of the AP but can be used to investigate changes in cellular physiology (such as elevated or reduced ion concentrations associated with disease states), the presence of 'unusual' electrical activity (such as is found with various arrhythmias resulting from myocardial ischaemia or infarction) or how drugs may interact with multiple ion channels concurrently and modulate or alter the AP.

In an actual heart, the situation is much more complicated than what can be described for a single isolated myocyte. Myocardial cells are coupled in three dimensions to adjacent cells as a means with which to assure the unified propagation of the AP and all cells assist in generating an AP synchronously. If this does not occur, disparity exists within the ventricle and altered excitability may result in disastrous consequences including proarrhythmia and sudden cardiac death. The uniform propagation of the AP is required for contractility and depends upon factors such as the geometric arrangement of cells, cell-to-cell coupling, the functional properties of each ion channel type involved in its genesis and current density within the cell membrane and its variation within regions of the heart.

5 The Cardiac Sodium Channel

Hodgkin and Huxley studied sodium conductance in the squid giant axon and were the first to propose a structural model for the channel such that the voltage-dependent opening and closing of membrane 'gates' resulted in a change in membrane permeability to sodium. The permeability change generated the AP and was responsible for the transmembrane movement of sodium ions. They proposed 'm' as an activation gate particle and 'h' as an inactivation gate particle, which display distinct kinetic properties highly dependent upon changes in membrane potential. Hodgkin and Huxley also postulated that for the conformational transitions of these gates to be voltage dependent, there must be a voltage sensor, or charge movement, during such transitions. They predicted the existence of the 'gating current'.

Depolarisation of the cell membrane opens Na^+ channels. However, this event only occurs after some delay. During this short time period (<1 ms), charge movement occurs. This delay was described as a series of voltage-dependent, closed-state conformational transitions the macromolecular protein which comprised the Na^+ channel had to pass through before the channel opened. The cause of this delay remained elusive until 1973 when Armstrong and Bezanilla first recorded the 'gating current'. It was a current of small amplitude (0.13 pA) and fast kinetics (80 μs to reach a maximum) (Armstrong and Bezanilla 1974). Thus, a majority of this current flows prior to the opening of the m gate for activation.

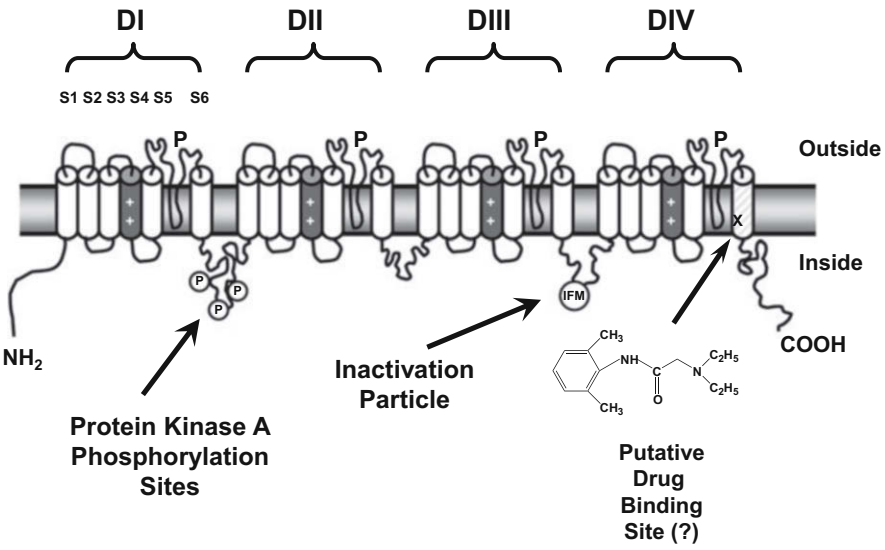

Fig. 2 A schematic of the proposed molecular structure of the Na^+ channel present in the heart. This model depicts the transmembrane folding of the primary structure of the Na^+ channel α-subunit. The domains of the channel are indicated as DI–DIV. The six α-helical transmembrane-spanning sequences are indicated as S1–S6 for the DI domain. Some experimentally determined sites for protein kinase A phosphorylation and local anaesthetic binding are shown. Those sequences involved in channel inactivation are also indicated along with those regions that constitute the ion channel pore (P)

5.1 Molecular and Pharmacological Properties

Molecular studies have revealed characteristics of the Na^+ channel itself as well as the putative 'voltage sensor' for the gating current (see Fig. 2). All voltage-gated Na^+ channels are comprised of approximately 2,000 amino acids and contain four homologous internal repeats (DI–DIV), each of which has six putative transmembrane (SI–S6) segments (Catterall 1993; Denac et al. 2000). The α-subunit is a phosphorylated and glycosylated protein that forms the ion channel pore and contains a particular region, the fourth transmembrane segment, S4, which is composed of a number of positively charged amino acids (lysine and arginine) and has been postulated to be the voltage sensor (Catterall 1993). Displacement by the change in membrane potential of these amino acids may be responsible for the gating current (Catterall 1995). The outward gating charge for sodium is due to the movement of 6 charges across the membrane. This finding corroborated the proposed charge displacement equivalent to 6 electrons flowing from the extra- to intracellular side of the membrane by Hodgkin and Huxley (1952). However, despite the implication of the S4 region in gating, the mechanism by which activation is initiated is not completely elucidated but does immediately precede Na^+ channel opening. The S6 regions within each of the four domains of the α-

subunit are thought to form the pore region of the channel while the P loops from each domain (see Fig. 2 for details) comprise the 'selectivity' filter in the outer or more extracellular region of the pore (Catterall 2001). Sato et al. determined, at a resolution of 19 A, the structure of the complete Na^+ channel in 2001. From a reconstruction of cryo-electron microscopic images, it is suggested that the Na^+ channel is bell shaped with the bulk of the protein structure (47 %) existing cytoplasmically. Structural features of the protein suggest an intercellular region for the inactivation particle and gating 'pores' for the outward movement associated with the voltage-sensing S4 transmembrane helices of each domain (Sato et al. 2001). These images also suggest that the central pore formed by the α-subunit is not simply linear across the membrane as has been suggested for decades but rather is divided into four branches that connect the cytoplasm to the extracellular medium (Sato et al. 2001).

Most Na^+ channels are heterotrimeric complexes in the membrane. The α-subunit ($\approx$260 kDa) interacts with at least 2 small auxiliary β-subunit proteins. The β_1-subunit ($\approx$36 kDa) is non-covalently associated with the α-subunit and has been shown to increase Na^+ current amplitude and increase the kinetic rate of activation and inactivation of the channel (Isom et al. 1992). While the expression of the α-subunit alone results in a functional channel, the β_1-subunit is responsible for refining the kinetic activity of the channel. These properties are crucial to the neuronal isoform of the channel but not the cardiac isoform of the channel. The β_2-subunit ($\approx$33 kDa) modulates Na^+ channel localisation in tissue. Various subtypes of voltage-gated Na^+ channels have been described (see Table 1) (Goldin 2001). The cardiac subtype is distinguished from most by being relatively insensitive to blockade by the puffer fish neurotoxin, tetrodotoxin.

Initially, local anaesthetics and anticonvulsant and antiarrhythmic drugs were believed to interact with the Na^+ channel and immobilise a fraction of the gating charge when the channel was blocked. However, Hanck et al. (1994) showed that drugs could bind the cardiac channel gate, but with altered kinetics, i.e. exhibit a reduced voltage dependency. In light of the Modulated Receptor Hypothesis (see below), this may alter our perspectives regarding the mechanism by which drugs interact with the Na^+ channel. Thus drug occupancy may reduce the voltage dependence of gating by inhibition of voltage-sensitive charge movement rather than by drugs producing a shift in channel states to the favoured drug-bound inactive state of the channel (Hanck et al. 1994).

Activation (or gating) is a change between a closed and resting (non-conducting) state of the Na^+ channel to an open (conducting) state in response to a change in membrane potential (see example of Na^+ current in Fig. 2). It occurs rapidly in excitable cells and is very steeply dependent upon depolarisation (Hille 1992). Thus, the rate of activation increases with membrane depolarisation. Activation of Na^+ currents generally occurs at thresholds between -40 and -60 mV via the voltage-dependent opening of the 'm' gate (see Fig. 3). The change in voltage opens the channel and allows for a rapid increase in Na^+ permeability. Activation kinetics can be altered by plant alkaloids such as veratridine, scorpion or sea anemone toxins or by insecticides such as pyrethroids (Catterall 1980). This kinetic property is not

Table 1 Various types of mammalian voltage-gated Na^+ channels

Tissue location	α-subunit	Channel[a]
Heart (human)	SCN5A	$Na_V1.5$
Heart/Denervated skeletal muscle (rat)	SCN5A	$Na_V1.5$
Heart/Uterine muscle (human)	SCN7A	$Na_V2.1$
Heart/Uterine muscle (mouse)	SCN7A	$Na_V2.3$
CNS (human)	SCN1A	$Na_V1.1$
CNS (human)	SCN2A1	$Na_V1.2$
CNS (human/mouse)	SCN8A	$Na_V1.6$
CNS (rat)	SCN1A1	$Na_V1.1$
CNS (rat)	SCN2A1	$Na_V1.2$
CNS (rat)	SCN3A	$Na_V1.3$
PNS (rat)	SCN8A	$Na_V1.6$
PNS (rat DRG)	SCN10A	$Na_V1.8$
Skeletal muscle (human)	SCN4A	$Na_V1.4$
Skeletal muscle (rat)	SCN4A	$Na_V1.4$

[a]According to the standardised nomenclature proposed for Na^+ channels at The IUPHAR/BPS Guide to PHARMACOLOGY website that builds upon and replaces the original IUPHAR Committee on Receptor Nomenclature and Drug Classification Database (IUPHAR-DB). See http://www.guidetopharmacology.org/ for details

DRG dorsal root ganglion

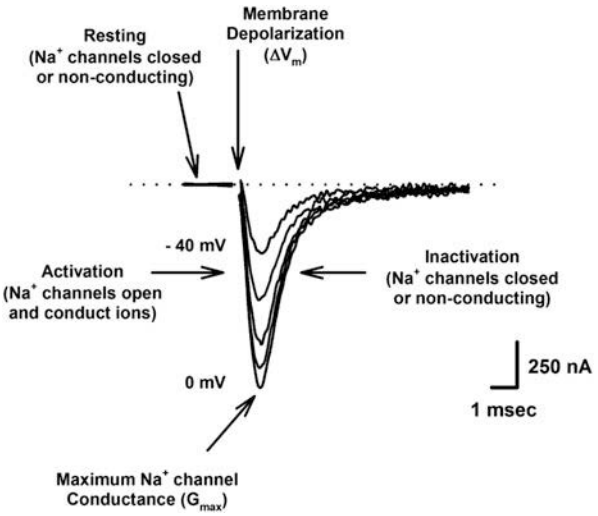

Fig. 3 A representative family of cardiac Na^+ currents traces evoked in *Xenopus laevis* oocytes. Currents were recorded by two-electrode whole-cell voltage clamp. Oocytes were injected with 50 ng of in vitro transcribed RNA encoding the SkM2 cardiac Na^+ channel α-subunit. After 2 days of incubation at 20 °C in ND-96 with supplements, currents were evoked by depolarising the cell to −40, −30, −20, −10 and 0 mV from a holding potential (V_m) of −120 mV at 3 s intervals. Maximal Na^+ channel conductance (G_{max}) was observed when the cell was depolarised to 0 mV. The evoked Na^+ channels all inactivated within ≈4 ms after depolarisation

altered by most antiarrhythmic or local anaesthetic drugs such as quinidine or lidocaine. These biological toxins bind to specific binding sites at extracellular sites on the Na^+ channel and generally shift activation to more negative membrane potentials. Thus, at resting membrane potentials a steady-state depolarisation results that is due to a sustained sodium current. It was originally proposed (for simplicity) that activation was independent of inactivation. However, it was not until Armstrong et al. (1973) perfused the squid giant axon with the enzyme pronase, and showed that inactivation was selectively destroyed and activation was unaltered and that the two processes could be dissociated.

Ionic conductance of the Na^+ channel is transient in nature. Prolonged depolarisation results in Na^+ channel inactivation and prevents the influx of Na^+ into the cell. Thus, refractoriness is maintained. As with activation, the rate of inactivation increases with an increase in the rate of depolarisation (see Fig. 3, panel b). Hodgkin and Huxley (1952) postulated that decay of Na^+ currents to resting values was mono-exponential. However, Chiu (1977) found that the rate of inactivation was much better approximated with a bi-exponential function and described two voltage-sensitive components for inactivation: fast and slow. Studies by Khodorov et al. (1976) described the slow component and how it was involved in cellular excitability but may have relevance in pathophysiological processes in brain and muscle tissue. Aldrich et al. (1983) used inactivation studies of single channel Na^+ currents to show that decay was biphasic, and largely coupled to activation, yet slow and fast inactivation are independent events. These studies indicate that some fraction of Na^+ channels must be open before inactivation proceeds (see Fig. 3). To date, slow inactivation, as a physiological process, remains poorly studied; however, fast inactivation has been extremely well investigated.

The inactivation gate, 'h', can be selectively destroyed by the internal application of protease and chemicals such as the piperazinyl-indole derivative DPI 201-106 (Wang et al. 1990). Veratridine and batrachotoxin, alkaloid toxins, also inhibit inactivation and produce a steady-state depolarisation due to enhanced sodium permeability (Catterall 1980). At the cellular level, this results in a prolongation of the AP and positive inotropism.

Molecular studies have shown that a highly conserved α-helical intracellular linker between domains III and IV (DIII–DIV) of the sodium channel (see Fig. 2) is responsible for fast inactivation kinetics (Goldin 1993). In addition, these molecular studies provide evidence for the proposed 'ball and chain' model of inactivation whereby this cytoplasmic linker may influence the activation and inactivation coupling process. This model suggests that a positively charged cytoplasmic protein particle (the 'h' gate using Hodgkin and Huxley formalism) electrostatically interacts with a negatively charged inactivation subunit of the sodium channel (Goldin 1993). Three amino acids (IFM) contained within the DIII–DIV loop sequence (isoleucine 1488, phenylalanine 1489 and methionine 1490) are crucial to channel inactivation (West et al. 1992) (Fig. 2). Thus, during inactivation this motif is thought to interact with amino acids that constitute a 'docking' site or

receptor within the pore region and block the pore, impeding the inward movement of Na^+ ions.

It is important that as a safety pharmacologist that one realises that any NCE in development is not unlike a local anaesthetic or classic antiarrhythmic drug interact with the inactivation gate (Hondeghem and Katzung 1977; Hille 1992). The inactivation produced by a change in membrane potential and drug block of the channel are interacting processes. These occur as a result of drug binding to a site on or near the 'h' gate in a voltage, time and channel state-dependent manner according to the Modulated Receptor Hypothesis (Hille 1992).

Hille in 1977 proposed a model for local anaesthetic action on nerve (Hille 1992). He suggested that there was a single specific binding site for local anaesthetics and that drug occupancy (block) alters the inactivation kinetics of the channel. The proposed location of drug action was intracellular. Hille also postulated that multiple pathways existed for drug access to these binding sites; thus, it could account for all drug access routes to this binding site. Hondeghem and Katzung (1977) used studies in cardiac muscle to propose a similar model for antiarrhythmic drug interaction with cardiac Na^+ channels. In this cardiac model, a series of equations were developed which defined binding parameters for each state of the channel (rest, open, inactive) and accurately described channel block by quinidine and lidocaine. The general model suggests that as Na^+ channels change states in a voltage-dependent manner local anaesthetic or antiarrhythmic drugs can associate or dissociate from each state. Thus, each state has a characteristic set of association (k) and dissociation (l) rate constants and voltage and time modulate binding (Hondeghem 1994). Since the affinity for the binding site is modulated by the state of the channel, the proposed model was called the Modulated Receptor Hypothesis (MRH) (Hille 1992; Hondeghem and Katzung 1977).

There is evidence for a specific binding site on the Na^+ channel for drugs. Ragsdale et al. (1996) identified a putative local anaesthetic, antiarrhythmic and anticonvulsant drug binding site on the S6 transmembrane-spanning region of domain IV (DIVS6) that lines the pore of the Na^+ channel (see Fig. 2). Additional delineation of the amino acids involved in drug binding suggests that pore-lining residues in the S6 region of domain III (DIIIS6) may also be involved in drug binding.

The existence of a persistent or 'late' Na^+ channel has been proposed for decades (Saint 2007). While the physiological role for the late Na^+ channel remains unknown, it has similar biophysical properties to the fast (or transient) Na^+ channel including ion channel selectivity and single channel conductance properties, but the population of ion channels that constitute the late channels fail to inactivate after opening (Saint 2007). The late Na^+ current is pharmacologically distinct and appears more sensitive to block by tetrodotoxin (TTX) and lidocaine as well as ranolazine and vernakalant (Ju et al. 1992; Saint et al. 1992). While the magnitude of the late Na^+ current in the normal heart is small (~1 % that of the fast Na^+ current), its magnitude is increased in many pathologic conditions including ischaemia (see Zaza et al. 2008). An enhanced cardiac late Na^+ current is proarrhythmic (i.e. elicits EADs, triggered arrhythmic activity, and TdP) an effect

that has been demonstrated in many SP assays including isolated Purkinje fibres, coronary-perfused wedge preparations and Langendorff hearts (Saint 2007; Zaza et al. 2008). This cardiac Na^+ current is currently being considered as a component to the CIPA ion channel assay.

With an understanding of the cardiac Na^+ channel complete, a return of the membrane potential to its pre-depolarising (resting) level begins with activation of Ca^{2+} current and repolarising K^+ currents.

6 Cardiac Calcium Channels

Voltage-gated Ca^{2+} channels are important regulators of electrical signalling and mechanical function in the heart. Calcium is an important ion to the myocyte (and to all cells) as it acts as an intracellular messenger in the initiation and/or regulation of many cellular processes including gene expression, enzyme function and cardiac muscle contraction.

At the myocyte level, Ca^{2+} is highly regulated both at the cell membrane and at intracellular loci (organelles) where concentrations $\approx 10^{-8}$ M are maintained. At the cell membrane, regulation is mediated by voltage-gated Ca^{2+} channels, by Ca^{2+} pumps and by the Na^+/Ca^{2+} exchanger. At intracellular loci, the sarcoplasmic reticulum, endoplasmic reticulum and mitochondria regulate Ca^{2+} levels. With such a complex hierarchical distribution and functional display by the Ca^{2+} ion, a number of voltage-gated channels exist that gate only this ion in excitable cells.

6.1 Molecular and Pharmacological Properties

Calcium channels are responsible for the genesis of APs in cardiac pacemaker cells (diastolic depolarisation) and the propagation of slow APs in sino-atrial and atrio-ventricular node cells and are equally important in the control of depolarisation-induced Ca^{2+} entry responsible for the plateau (phase 2) of the AP (see Fig. 1).

Voltage-gated Ca^{2+} channels are hetero-oligomeric protein complexes that are comprised of an α_1- ($\approx$240 kDa) subunit, a β-subunit ($\approx$60 kDa) and an accessory $\alpha_2 - \delta$ ($\approx$175 kDa) subunit (Striessnig 1999). At least six classes of voltage-gated Ca^{2+} channel have been characterised. Cloning studies reveal that these channels exist in various tissues as a result of the co-assembly into protein complexes with variable accessory subunits (see Table 2). Expression in heterologous systems has allowed for their electrophysiological and pharmacological characterisation. There is a single L-type and five non-L-type channels called T, N, P/Q and R (Ertel et al. 2000). These channels can be further subdivided into those that exhibit low voltage-activating (LVA) properties and rapidly inactivate (properties of the T-type channel isoform) and those that are highly voltage-activated (HVA) and do not inactivate.

Each of the α_1-subunits of the Ca^{2+} channel, composed of approximately 1,800 amino acid residues, is the major protein constituent that contains the ionic pore, the

Table 2 Types of mammalian voltage-gated calcium channels

Tissue location	α-subunit	Gene	Channel
Heart (rat)	α_{1C}	CACNA1C	L-type
Heart (rat)	α_{1G}	CACNA1G	T-type
Heart (human)	α_{1H}	CACNA1H	T-type
CNS/NMJ	α_{1A}	CACNA1A	P/Q-type
CNS/NMJ	α_{1B}	CACNA1B	N-type
CNS/NMJ	α_{1E}	CACNA1E	R-type
Retina	α_{1F}	CACNA1F	L-type
Skeletal muscle	α_{1S}	CACNA1S	DHP

Note: L- and T-type channels are found in cardiac muscle where they are sensitive to blockade by pharmacological agents including verapamil and diltiazem

selectivity filter, intracellular regulatory regions and necessary gating machinery of the channel (see Fig. 4). Ten α_1-subunit genes have been identified as $\alpha_{1A} - \alpha_{1I}$ and α_{1S}. In cardiac ventricular muscle, only the α_{1C}-subunit encoding the L-type Ca^{2+} channel is found at appreciably high levels (>80 %) while α_{1D}-subunit expression is found in atrial muscle (Striessnig 1999; Ertel et al. 2000). Of the three isoforms of the α_1-subunit that encode for the T-type channels (see Table 2), only α_{1G} and α_{1H} are found in cardiac tissue.

The α_1-subunit also contains the binding domain for Ca^{2+} channel antagonist drugs. The L-type channel, which carries I_{si} in the heart, is blocked by three groups of drugs. The phenylalkylamine (e.g. verapamil) and benzothiazepine (e.g. diltiazem) blockers are effective clinically used antiarrhythmics while the 1,4-dihydropyridines (e.g. nifedipine) are useful antihypertensive agents. Chemically, Ca^{2+} channels show a marked structural homology to each other and to voltage-gated Na^+ channels. This subunit is composed of four homologous domains (DI–DIV), each of which is composed of six transmembrane-spanning α-helical proteins that form a pore in the membrane (see Fig. 4). Like Na^+ channels, the fourth transmembrane helix (S4) contains positively charged amino acids which are responsible for the gating or voltage-sensing activity of the channel. The sixth transmembrane helix of each domain is responsible for conferring inactivation properties to the channel.

Calcium channels, like many other voltage-gated ion channels, require auxiliary subunits for functional expression. Currently four mammalian isoforms of the β-subunit exist. These polypeptides vary between 52 and 71 kDa and are involved in membrane stabilisation and trafficking of the α_1-subunit within the cell (DeWaard et al. 1994). Note that the cardiac L- and T-type Ca^{2+} channels are only co-expressed with the β_2-subunit isoform. Of the three $\alpha_2 - \delta$ subunit isoforms that have been detected in various tissues, only the $\alpha_2 - \delta_1$ and $\alpha_2 - \delta_2$ types are expressed in the heart (Klugbauer et al. 1999). The α_2 component is a highly glycosylated extracellular protein that associates with extracellular regions of the α_1-subunit and stabilises drug binding while the δ component may stabilise channel gating properties (Striessnig 1999).

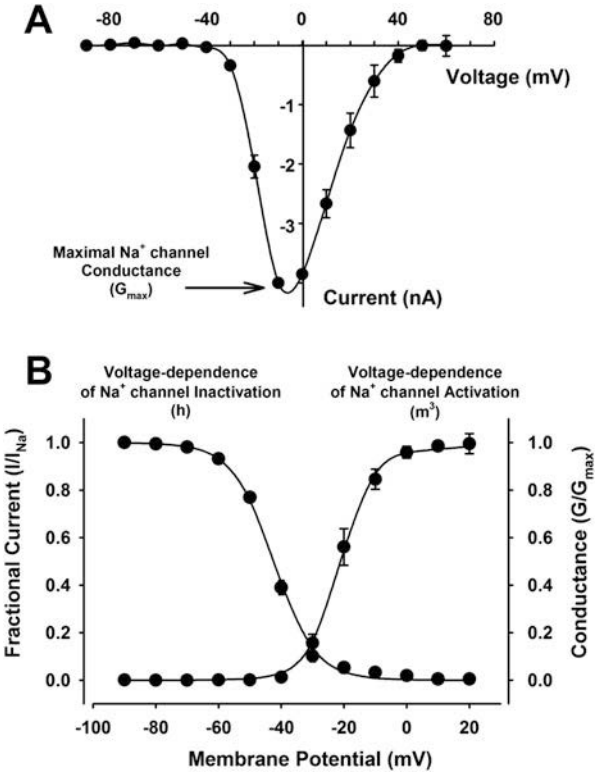

Fig. 4 Biophysical properties of cardiac voltage-gated Na^+ ion channels. Panel (**a**) depicts the current–voltage relationship for the cardiac rH1 Na^+ current isoform expressed in *Xenopus* oocytes. Cells were held at a membrane potential of −120 mV and currents were measured by depolarisations ranging from −90 to +50 mV in 10 mV increments. Sodium channel conductance (G_{max}) was maximal at a depolarising potential to −10 mV. The data are plotted as peak current amplitude versus the pulse potential and the curve for the figure is the best fit of the equation: $I = G_{max}/1 + \exp[(V - V')/k]\ (V - E_{rev})$ (see text for details). Panel (**b**) shows the voltage dependence of Na^+ channel activation (m^3) and inactivation (h) for the cardiac rH1 isoform expressed in oocytes. The voltage dependence of Na^+ channel activation or conductance (G/G_{max}) was calculated by measuring the peak current at test potentials ranging from −90 mV to +20 mV evoked in 10 mV increments and dividing by $(V - V_{rev})$, where V is the test potential and V_{rev} is the reversal potential for Na^+. Peak conductance values were fit with a two-state Boltzmann equation of the form $G = 1*[1 + \exp(-0.03937 \cdot z_{app} \cdot (V - V_{½})]$. The voltage dependence of steady-state inactivation was determined using 500 ms conditioning pre-pulses from a holding potential of −120 mV to +15 mV in 10 mV increments, followed by a test pulse to −5 mV for 22.5 ms. The peak current amplitude evoked during the test depolarisation was normalised to the maximum current amplitude and plotted as a function of the conditioning pre-pulse potential. The data were fit with a two-state Boltzmann equation of the form $I = I_{max}*[1 + (\exp(V - V_{½})/k)]^{-1}$

As mentioned above, the α_1 isoforms determine the pharmacological properties of each of the Ca^{2+} channels in the heart. The L-type Ca^{2+} channels possess high-affinity, stereoselective-binding domains for channel blocking drugs, and thus blockade of these Ca^{2+} channels in the heart exerts antiarrhythmic activity against

supraventricular arrhythmias. A combination of complementary photo-affinity labelling, antibody mapping and cloning studies of the various α_1-subunits present in the heart suggests that it is the S6 regions of domains III and IV and the S5–S6 linker of domain III that may contain the actual high-affinity binding sites for channel blocking drugs. All Ca^{2+} channel blocking drugs bind with close proximity to the pore and as a result of their binding alter the actual binding site for Ca^{2+} ions within the pore (Fig. 4). These actions have been used to explain the non-competitive allosterism that is observed for various blocking drugs on many Ca^{2+} channel preparations. The binding of channel blocking drugs is suggested to produce conformational changes in amino acids in this region, which then alter pore-associated binding properties to Ca^{2+} ions.

Unlike the L-type cardiac Ca^{2+} current, the T-current rapidly inactivates and is involved in pacemaker activity in the sino-atrial node and intracellular Ca^{2+}-induced Ca^{2+} release.

7 Cardiac Potassium Channels

7.1 Diversity of Voltage-Gated Potassium Channels

During the 1980s and into the 1990s, interest in the development of drugs which prolong refractoriness, i.e. possess class III antiarrhythmic action, had increased markedly. Several reasons for this resurgence in interest included the negative results of the CAST trials where proarrhythmic tendencies were associated with some class I agents and the effectiveness of long-term studies with amiodarone which suggested that it may, in a manner similar to the β-blockers, decrease post-infarction arrhythmic death (Vaughan Williams 1984). Repolarisation and the configuration of phase 3 of the AP in cardiac tissue occur as a result of the complex interaction of multiple K^+ channels (Snyders 1999). These K^+ channels are heterogeneous and differ in gating and permeation properties as well as in susceptibility to modulation by neurotransmitters, intracellular ions such as Na^+ and Ca^{2+} and NCEs. In essence, K^+ channels regulate cell function by establishing the resting membrane potential and controlling cell repolarisation processes. Individual K^+ currents overlap in their contribution to the total membrane current during the AP. The relative importance of each may vary under different conditions; for example, during ischaemia changes in cell electrophysiology may alter the degree to which different channels contribute to the AP.

Amongst ion selective channels within the myocardium, the K^+ channel is unrivalled in terms of molecular and functional diversity. As the genetic, molecular and functional diversity of K^+ channels grows so does the general complexity with regard to its pharmacology and nomenclature. In general, mammalian K^+ channels have been categorised into three main families: the voltage-gated K^+ channels (K_v), the inward rectifying K^+ channels (K_1) and the two pore domain channels (K_{2P}) (see Table 3). However, the literature is rife with creative modifications to this simple classification scheme. For the purposes of the present discussion, channels will be

Table 3 Types of mammalian cardiac voltage- and ligand-gated potassium channels

Cardiac current	α-Subunit clone
Transient outward (I_{to})	
Fast component ($I_{to,f}$)	$K_v4.2/K_v4.3$
Slow component ($I_{to,s}$)	$K_v1.4$
Delayed rectifier (I_K)	
Rapid component (I_{Kr})	$K_v11.1$ (hERG)
Slow component (I_{Ks})	K_vLQT1 + min K
Ultra-rapid component (I_{Kur})	$K_v1.5$
Inward rectifier (I_{K1})	
I_{K1}	Kir2.1
(I_{KATP})	Kir6.2 + SUR2A
(I_{KAch})	(GIRK) Kir3.4 + Kir3.1
Pacemaker current (I_f)	HCN4

described using the classification system mentioned above, but safety pharmacologists should be aware of alternate names for these channels. Refer to the IUPHAR/BPS Guide to PHARMACOLOGY website for the latest detailed list of ion channel nomenclature (Pawson et al. 2014).

Genes that encode the nucleotide sequence(s) responsible for the expression of functional K^+-selective ion channels in the myocardium are distributed widely throughout the genomes of many diverse species. These include yeast (*S. lividans* and *S. cerevisiae*), the nematode *C. elegans*, the fruit fly (*D. melanogaster*), the cyanobacteriae (*A. thaliana*), the mouse (*M. musculus*) and humans (*H. sapiens*). In some instances, the gene sequences and functional characteristics of the channels themselves are highly conserved across species (MacKinnon et al. 1998). Note that mutations in K^+ channel genes may be responsible for certain types of cardiac pathology that may or may not be dependent upon environmental factors (e.g. idiopathic long QT syndrome and hypokalemia) (Roberts and Brugada 2000).

In general, all K^+ currents have a similar primary amino acid sequence with highly conserved structural regions (see Fig. 5). The molecular structures of K^+ channels may be described as having one or two pore-forming domains and two, four or six transmembrane-spanning domains (collectively referred to as the α-subunit). The molecular diversity of K^+ channels, like the Ca^{2+} channels described above, is largely due to variability in the heteromeric association of pore-forming α-subunits and accessory, or β-subunits, during formation of channel complexes (Nerbonne 2000). Voltage- and Ca^{2+}-dependent K^+ channel α-subunits exhibit six transmembrane-spanning sequences with a voltage sensor in S4 and a pore-forming region located between S5 and S6 (see Fig. 6). Inwardly rectifying K^+ channel α-subunits are comprised of two transmembrane-spanning sequences and like the voltage-gated channels have one pore-forming region. Other K^+ channels that exhibit little or no voltage-dependent action but are modulated by pH, stretch, temperature and a variety of second messengers are formed through the dimerisation (sometimes requiring a cysteine residue) of α-subunits containing two pore-forming sequences and four transmembrane sequences. The common

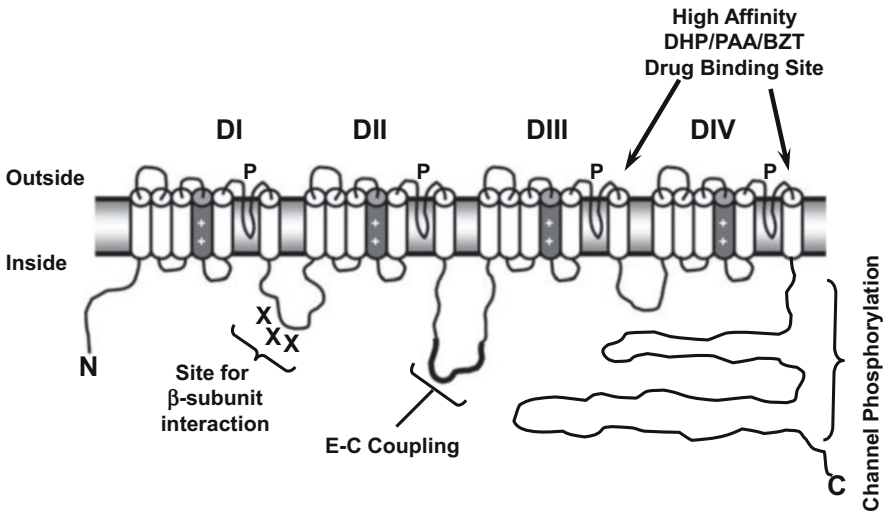

Fig. 5 A schematic of the proposed molecular structure of the Ca^{2+} channel present in the heart. This model depicts the transmembrane folding of the primary structure of the Ca^{2+} channel α-subunit. The domains of the channel are indicated as DI–DIV. The six α-helical transmembrane-spanning sequences are shown for each domain. Transmembrane segment S4, the channel voltage sensor, is depicted with (+) residues and those regions associated with formation of the channel pore as P. Some experimentally determined sites for β-subunit interaction, channel phosphorylation and excitation–contraction (EC) coupling are shown along with the putative α-helices important for the high affinity of dihydropyridine (DHP), phenylalkylamine (PAA) and benzothiazepine (BTZ) drugs

feature amongst these different channel-forming strategies is that the ion selective pore is always formed by the fusion of four pore-forming segments. Also, K^+ channels all commonly contain regulatory sites on both amide and carboxyl terminal sequences. Similar sites are also located on the β-subunits that in turn directly modify α-subunit function (Nerbonne 2000).

The functional properties of currents carried by K^+ channels range from those with strong time- and voltage-dependent activation and inactivation kinetics with an associated strong inward or outward rectification (e.g. as seen with I_{Kr}) to time- and voltage-independent activation and inactivation kinetics with very weak rectification (e.g. as seen with $I_{TWIK\text{-}1}$). Voltage-dependent activation of K^+ currents plays a considerable role in the repolarisation of the cardiac cell membrane whereas constitutive activation of K^+ currents that are voltage *independent* may play roles in both repolarisation and maintenance of the cell resting membrane potential. Voltage-dependent inactivation may proceed either rapidly or slowly by N- and C-type inactivation, respectively (Rasmusson et al. 1998). Cardiac K^+ channels that activate and inactivate rapidly (such as the fast component of the transient outward K^+ current, $I_{to,f}$), slowly (such as the slow component of the delayed rectifier K^+ current, I_{Ks}) or at variable rates (such as the ultra-rapid component of the delayed

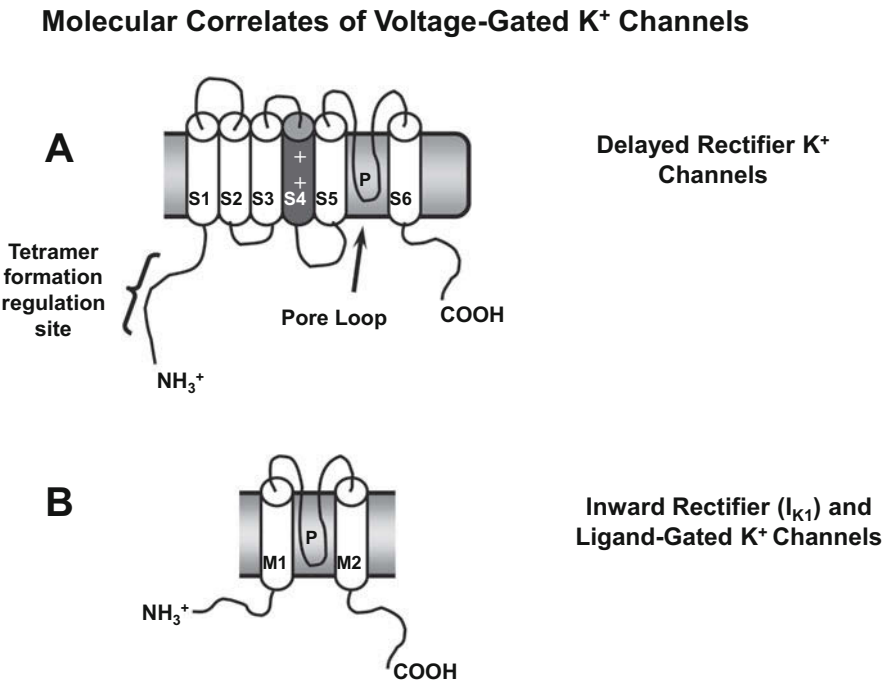

Fig. 6 Molecular correlates of some native voltage- and ligand-gated K^+ channels found in cardiac muscle. Panel (**a**) depicts the putative molecular model shared by delayed rectifier (I_K) and transient outward (I_{to}) K^+ channels. These subunits contain six transmembrane segments (S1–S6) and one P-loop for the mammalian channel clones $K_v1.x - K_v4.x$ (I_K and I_{to}), HERG (I_{Kr}) or K_vLQT1 + minK (I_{Ks}). Panel (**b**) depicts subunits that encode the inward rectifier (I_{K1}) and several ligand-gated K^+ currents including I_{KATP} and I_{KAch}. These channels contain only two transmembrane segments (M1 and M2) and one P-loop for the mammalian channel clones Kir2.1 (I_{K1}), Kir6.2 + SUR1 (I_{KATP}) and Kir3.1 + Kir3.4 (I_{KAch})

rectifier K^+ current, I_{Kur}) will therefore contribute differently to cardiac cell excitability and repolarisation under different circumstances (e.g. at fast or slow heart rates). Potassium channels may have very diverse mean open times such that some channels may be open as briefly as 1 ms or remain open for hundreds of milliseconds. Single channel conductance through the open cardiac K^+ channel may be low in some channels (such as <5 pS in the two-pore domain K^+ channel I_{TWIK-2}), moderate in others (such as 20–30 pS in the transient outward K^+ channel, I_{to}) and high in others (such as >100 pS in the TWIK-related K^+ channel I_{TREK-1}). Note however that ion channel conductance depends upon the expression system employed (i.e. is the system native or heterologous or involve stem cell differentiation and expression?) and the recording environment (i.e. currents should be recorded using the whole-cell configuration of the patch clamp and utilise physiologically relevant K^+ concentrations used to delineate channel activity). Such criteria should be strictly defined in any SP electrophysiology study and strive

for physiological relevance in order to optimise biophysical properties of K^+ channels in cardiac tissue to best characterise any potential for interactions with NCEs.

Currents carried by K^+ channels are therefore extremely important in the regulation of myocardial cell resting potential and repolarisation and, thus, to the configuration of the cardiac AP and ECG morphology. The differential distribution of K^+ channels within the myocardium accounts for the large variation in K^+ current density, AP morphology within different cells of the heart and in AP and ECG morphology across species (Yan and Antzelevitch 1996; Yan et al. 1998). The abundance of K^+ channel mRNA can vary dramatically in different cell types (see below for more detail). The combined effects of highly varied K^+ channel type and subtype expression, function and regulation converge in the myocardium to produce a complex effect on the electrical excitability of cardiac cells and the morphology of recorded electrical activity in normal, diseased and drug-treated hearts.

Traditionally K^+ channels have been characterised pharmacologically based on their sensitivity to tetraethylammonium (TEA), 4-aminopyridine (4-AP) or cations such as cesium (Cs^+) and barium (Ba^{2+}). More recently, naturally occurring venoms such as that isolated from the scorpion and synthetic compounds including dofetilide, astemizole, E-4031, JNJ-202 and Chromanol 293B have been used to successfully isolate and study specific components of different K^+ channel currents. While K_v channels are ubiquitously sensitive to blockade by either TEA or 4-AP, they exhibit a differential sensitivity to a range of cardiovascular and non-cardiovascular drugs in normal (and diseased) myocardium (Yang and Roden 1996).

The inward rectifying K^+ channels (K_{ir}) couple direction of K^+ flow in the myocyte (inward *against* the normal K^+ ion concentration gradient) to the metabolic status of the myocardium. These channels are commonly activated or blocked by the products of cellular metabolism and by various cations. The K_{IR} family is composed of a large number of channel subtypes; some are voltage dependent while others are not. In addition, some channels in this group are ATP sensitive (I_{KATP}) or GTP activated. Many synthetic and naturally occurring drugs can activate (benzopyrans) or block (sulfonylureas) I_{KATP} currents in cardiac and non-cardiac (pancreatic) tissue providing these channels as good clinical targets for drug development.

The safety pharmacologist should be aware of the differences that single or multiple nucleotide polymorphisms (SNP) make in the gene sequences of various K^+ channels that result in the expression of ion channel proteins with either severe or subtle changes in biophysical properties. While these proteins may perform most functional tasks sufficiently such that myocyte function is not significantly impeded, drug properties may change and hence unanticipated clinical adverse events such as prolongation of the QT interval could occur or precipitate other untoward adverse responses of the myocyte to drugs in the clinical population. Therefore changes in K^+ channel structure can contribute to a number of congenital or drug-induced cardiac disorders including the long QT syndrome, idiopathic

polymorphic VT, Andersen's Syndrome and the Jervell and Lange-Nielsen Syndrome.

Thus, the heterogeneity of K^+ channels provides for a large potential for NCEs in development to potentially block K^+ channels. This is well documented within the drug safety literature where the therapeutic potential and benefits of these agents were dashed by their ability to precipitate TdP arrhythmias (Pugsley 2002).

An important property that should be understood when assessing the safety profile with regard to blockade of K channels is that prolongation of the APD occurs optimally at low rates of stimulation while are limited at high heart rates. This 'reverse use dependence' is an important drug property to characterise with any drug as it suggests that mechanistically these drugs may have a high affinity for the closed state of the K^+ channel (Pugsley 2002). Only amiodarone lacks this effect. The resulting bradycardia associated with potent and selective I_{Kr} channel blockade is known to precipitate arrhythmias such as the prolonged Q–T and TdP.

Of the many K^+ channels that exist in cardiac muscle, we will provide an overview of only those K^+ conductances which carry most of the outward repolarising current and that are important in understanding the basis for development of the CIPA paradigm for SP. These include only the transient outward (I_{to}) K^+ current, the delayed rectifier K^+ (I_K) current and its components that contribute predominantly during the plateau and early stages of repolarisation of the AP and the inward rectifier (I_{KIR}) K^+ channels.

7.2 Voltage-Dependent (K_V) Transient Outward (I_{to}) and Delayed Rectifier (I_K) Channels

The voltage-dependent K^+ currents (K_V) that have the greatest influence on changes in cardiac cellular resting membrane potential, especially repolarisation, are the transient outward (I_{to}) and the delayed rectifier (I_K) K^+ currents and their many subtypes (Nerbonne 2000). K_V channel α-subunits are derived from six families of channel proteins, most of which are based on the *D. melanogaster* nomenclature (*Shaker*, *Shab*, *Shaw*, *Shal*, *Eag* and K_VLQT1). The genes responsible for the mammalian cardiac α-subunits that produce the pore-forming regions and some of the β-subunits that modify channel expression and function are distributed widely throughout the genome. In most cases, the K_V genes are transcribed according to the same mechanisms that operate for transcription of other voltage-dependent ion channels. Large conductance Ca^{2+}- and voltage-activated K^+ channels are also found within the myocardium; however, these ion channels are predominantly expressed in neurons and vascular smooth muscle cells and will therefore not be discussed here.

7.2.1 Molecular Genetics of I_{to} and I_K Channels

All K_V channels are comprised of four α-subunits and may co-assemble with at least one of four possible β-subunits. K_V α- and β-subunits co-assemble within the endoplasmic reticulum and exhibit chaperone-dependent trafficking to the

membrane surface (Kuryshev et al. 2001). Each α-subunit (see Fig. 5 for details) consists of six transmembrane-spanning sequences (S1–S6) that perform a variety of functions including voltage sensing (the S4 region), rapid N-type inactivation (the N-terminal and S5 region), slower C-type inactivation (the H5 pore loop and S6 region) and intracellular communication (the C-terminal) (Armstrong and Hille 1998). K_V channels are unique compared to their inward rectifying counterparts in that functional channels may form through the heteromeric association of α-subunits and that subunit dosage may regulate membrane trafficking of functional channels. The molecular correlates of heteromeric α-subunit channels have been the subject of intense investigation in a number of tissues and in a variety of species (Nerbonne 2000).

The transient outward K^+ channel (I_{to}) is a relatively common current found in a wide variety of species and cell types. The channel possesses rapid activation and inactivation kinetics and is important during early phase I repolarisation (Coraboeuf and Carmeleit 1982). It is coupled to both Na^+ and Ca^{2+} and is critical for the classic 'spike and dome' appearance of the ventricular AP. Channel density and distribution differences amongst cell type's results in a variable AP morphology in various regions of the heart. I_{to} are composed of a large voltage-activated, Ca^{2+}-independent component, $I_{to,f}$ (or I_{to1}), and a small Ca^{2+}-activated component, $I_{to,s}$ (or I_{to2}), and the channel activates at membrane potentials more positive than -70 mV and inactivates at -10 mV (Coraboeuf and Carmeleit 1982). I_{to} is highly K^+ selective, shows little rectification, reaches its peak in 3 ms and, depending on the species, shows either mono- or bi-exponential rates of inactivation (Jahnel et al. 1994). In the rat, I_{to} is the main repolarising current and is sensitive to blockade by 4-aminopyridine (4-AP) and tedisamil (Beatch et al. 1991).

The fast component of the transient outward current ($I_{to,f}$) is comprised of predominantly $K_V4.2$ and $K_V4.3$ α-subunits; however, it depends upon the selection of cardiac tissue whereas the slow component ($I_{to,s}$) is primarily composed of $K_V1.4$ α-subunits (Wickenden et al. 1999). Modulation of both I_{to} and I_K currents by accessory β-subunit proteins is important, especially in many cardiac pathophysiological diseases.

The time- and voltage-dependent K^+ current (I_K) channel, also called the 'delayed rectifier', is governed by a single voltage-dependent gate that activates slowly. The G_{max} for this channel shows some slight 'inward rectification', i.e. the current decreases markedly with depolarisation. This rectification is in the opposite direction to that predicted by the GHK current equation. This current is dependent upon extracellular K^+ and because the channel has appreciable Na^+ permeability V_K is less negative than the Nernst potential for K^+. The slowness of activation of the I_K channel may be regarded as permitting the prolonged plateau of the cardiac AP (phase 2), despite slow inactivation of voltage-dependent Ca^{2+} channels and its eventual activation as principally responsible for terminating phase 2. The sensitivity of I_K to extracellular K^+ accounts very largely for the phenomenon whereby phase 2 is prolonged (and repolarisation, phase 3, delayed) when extracellular K^+ is lowered, and the reverse when it is raised. The delayed rectifier K^+ current (I_K) has (at least) two components, one relatively large and slow to activate (I_{Ks}) and the

other small and more rapidly activating (I_{Kr}). Currently a number of I_K channel types have been cloned and are comprised of many channel subtypes that result from the functional assembly of $K_V1.1$, $K_V1.2$, $K_V1.5$, $K_V2.1$, $K_V2.2$, $K_V3.1$, K_VLQT1 and HERG α-subunits (Nerbonne 2000).

7.2.2 Functional and Pharmacological Properties

The functional properties of most K_V channels have been described in detail in native and heterologous expression systems and the effects of modifications to various regions of the S1–S6 transmembrane sequences of the α-subunits have been well characterised (Boyett et al. 1996). Both of the I_{to} channel subtypes activate early and the distinguishing functional feature that arises between $I_{to,f}$ and $I_{to,s}$ current involves their rates of channel inactivation and recovery from inactivation. While I_{κ} currents activate at variable rates, they all tend to inactivate slowly, or very slowly, with the inactivation kinetics of I_{Kr} being the only exception and a unique identifier of that current. Native and heterologously expressed $K_V4.2$ and $K_V4.3$ channels exhibit sensitivity to protein kinase C (PKC) that is manifest as a reduction in outward K^+ current due to changes in inactivation kinetics and recovery from inactivation (Nakamura et al. 1997). The voltage-dependent, ultra-rapidly activating K^+ current (I_{Kur}) is sensitive to protein kinase A (PKA)-mediated phosphorylation and it is known that the PKA regulation of $K_V1.5$ channel function is mediated by a specific set of N-terminal residues on the $K_V\beta1.3$ subunit (Kwak et al. 1999). In native atrial myocytes α- and β-receptor mediated adrenergic stimulation may significantly modify members of the I_K family such as I_{Kur} via interactions with PKC and PKA (Yue et al. 1999). Therefore, K_V channels are not only unique with regard to their regulation by voltage but also exhibit sensitivity to modification by the intracellular and extracellular myocardial environment, a sensitivity that may enhance the potential for pharmacological regulation of K_V channels.

Like the functional properties of K_V channels, the pharmacology of native and cloned K_V channels has been described in considerable detail (Rolf et al. 2000. However, one should note that there are key aspects of K_V channel pharmacology which produce significantly different effects under either normal or pathological cardiac conditions. It remains very difficult to isolate specific channel subtypes in vitro due to (1) contamination of preparations by other K^+ currents, (2) poor selectivity of subtype-specific blocking agents and (3) the inability to measure gross electrophysiological variables (APD, ERP) using cloned channels in heterologous expression systems. The reverse use dependence associated with drugs interacting with K^+ channel has been rigorously investigated. Reverse use dependence is a pronounced pharmacological phenomenon associated with K_V channel block and should be an electrophysiological parameter of assessment in drug safety studies.

Pharmacologically, $I_{to,f}$ and $I_{to,s}$ can be distinguished by their sensitivity and insensitivity, respectively, from the class Ic antiarrhythmic drug, flecainide and the heteropodatoxins (Xu et al. 1999). The pharmacology of K_V channels is complex. Several examples of the complexity are given. The rapid (I_{Kr}) and slow (I_{Ks}) activating components of I_K are readily distinguishable based upon their sensitivity and insensitivity, respectively, to methanesulfonamide drugs such as dofetilide and

E-4031 and the cation La^{3+} (Main et al. 1998). I_{Kur} or the $K_v1.5$ channel is present in human atrial tissue. It is outwardly rectifying and highly selective for K^+. Like other K_V channels it activates during the plateau phase of the cardiac AP and is distinguished from related K_V channels by its sensitivity to very low concentrations of extracellular 4-AP. $I_{K,slow}$ is another distinct K_V channel represented by $K_v1.2$ expression (Nerbonne 2000). It is sensitive to block by very low concentrations of 4-AP but unlike I_{Kur} this current is sensitive to nanomolar concentrations of dendrotoxin (DTX) and thus may be designated as $I_{K,DTX}$ (Nerbonne 2000).

As a result of its rapid voltage-sensitive activation and inactivation, $I_{to,f}$ contributes substantially to the very early phase of repolarisation prior to the AP plateau. It exhibits marked, variable expression patterns in cardiac tissue. These differences in gradients for the expression of $K_v4.2$ proteins exist at all levels in the heart including transmurally where $I_{to,f}$ is present at high levels in the epicardium and at low levels in the endocardium, interventricularly where it is present at greater levels in the right compared to the left ventricle, intraventricularly where $I_{to,f}$ is present in the apex and septum, but $I_{to,s}$ is present in the septum only, and longitudinally where $I_{to,f}$ is greater in the apex than the base of the heart. These differences therefore account for the sharp spike and dome appearance of APs obtained from the epicardium compared to the endocardium as well as for differences in the spike and dome morphology in epicardial cells from different species and may account for the varied J–T interval duration and S–T segment elevation observed between species. For example, ventricular $K_V4.2$ subunit levels are greatest in the rat and non-existent in the guinea pig (rat > dog > human >>> guinea pig) (Nakamura et al. 1997; Gussak et al. 2000). Interestingly, small and large mammals may regulate the expression profiles of functional K_V4 channels via different mechanisms.

Like K_V4 channels, K^+ channels that carry the many varieties of I_K currents also exhibit differential expression profiles within the mammalian myocardium. In dogs and humans, I_{Kr} densities are larger in left versus right atrium whereas I_{Ks} current densities exhibit an even distribution across human atria (Li et al. 2000). Different inter-atrial distributions of I_K may have a functional role in the maintenance of re-entry arrhythmias (Li et al. 2000). Gintant (1995) demonstrated transmural differences in I_K current densities that are associated with longer APDs in mid-myocardial cells of dogs as compared to epi- and endocardial cells. In guinea pigs, ventricular subepicardial myocytes and M-cells exhibit the longest and shortest APD_{90} values and the lowest and highest I_K densities, respectively (Main et al. 1998). Expression of I_{Kur} in dogs is limited to atrial tissue and may therefore be responsible for shorter APD observed in canine atrium versus ventricle (Yue et al. 1999).

Due to the strong rate-dependent activation and inactivation kinetics displayed by K_V channels and the heterogeneity of K_V channel distribution within the myocardium, the combined effect of K_V channel expression and function on AP morphology will vary considerably under normal and abnormal conditions and between species and are therefore difficult to translate into specific effects on the ECG. However, differences in ECG morphology between species at rest under

normal conditions and under abnormal or diseased states do allow us to make some generalisations based on the difference in functional effects of K_V channels observed under experimental conditions.

Unfortunately atrial repolarisation is masked in the surface ECG by ventricular depolarization. However, effects of drugs and pathology on ventricular repolarisation can be mapped on the surface ECG through analysis of the J–T segment of the ECG. Under normal circumstances, direct reductions in K_V-mediated current are expected to prolong ventricular repolarisation and thereby produce a prolongation of the QT and J–T segments of the ECG. There is also an increase in the height of, and area under, the T-wave and possibly production of an abnormal T-wave morphology. Guo et al. (2000) have demonstrated that transgenic mice expressing homozygous dominant negative K_V4.2 and K_V1.4 subtypes of I_{to} exhibit rate-dependent prolonged ventricular AP and QT intervals and also exhibit spontaneous ventricular tachyarrhythmias. These same authors demonstrated that K_V1.4 elimination alone had no significant effect on QT interval duration and that K_V4.2 alone and in combination with K_V1.4 elimination had profound effects on QT interval duration, thereby suggesting that compensatory upregulation of I_{to} subtypes may occur and thereby act as a limiting factor to excessive QT interval prolongation. In a mouse model of the Jervell and Lange-Nielsen syndrome in which a double homozygous loss-of-function mutation in KCNQ1 is introduced, animals exhibit the three characteristics described above (Casimiro et al. 2001). It should be recognised that KCNQ1 was the first member of the KCNQ family of K^+ channels that are structurally similar to K_V and is identified as the gene responsible for the long QT syndrome, LQT1. Thus, under relatively defined conditions, reductions in K_V channel function and expression have potentially predictable effects on APD and the ECG.

It should be appreciated by safety pharmacologists that as the NCE drug concentration increases in the model or plasma, cellular effects become less defined due to the complex interactions between the NCE and many ion channels, their multiple subtypes and activation of compensatory and adaptive physiological mechanisms. Under these circumstances, the expression and function patterns of a single ion channel group or subgroup may not produce the predicted macroscopic effects as measured by either the AP or ECG. For example, both I_{to} and I_K are reduced (>50 %) in chronic and postoperative atrial fibrillation and both disorders are marked by reduced atrial APD and atrial ERP (AERP) and maintained re-entry, a consequence of AF-induced remodelling. An understanding of the properties of the ion channels involved would suggest that reductions in these currents should produce an anticipated contradictory effect. That is, APD prolongation and an increase in AERP followed by an increase in re-entry path length and abolition of the arrhythmia should result. However, in reality the reductions in I_{to} and I_K probably do serve as protective measures but are impeded in their efforts by changes in other ion channel function. It is known that a reduction in I_{Ca} may be greater than the overall reduction in I_K in atrial fibrillation (AF) and therefore may have a greater impact on APD and AERP reductions associated with chronic and postoperative AF. This concept has been reiterated by work in dogs that shows that

reductions of up to 75 % of atrial I_{to} current could not compensate for reductions in I_{Ca} associated with AF and remodelling (Yue et al. 1997). However, the effects described above appear to be very specific for AF secondary to Ca^{2+} overload-induced hypertrophy and the physical act of remodelling under the influence of the arrhythmia. Therefore, location and distribution of K_V channels and their interactions with other ion channel families may have important implications for specific diseased states, pharmacological management and safety.

Heart failure, hypertrophic cardiomyopathy and myocardial infarction are just several of the cardiac disorders associated with prolonged and abnormal QT intervals of the ECG and region-specific reductions in I_{to} and I_K with subsequent increases in APD (Yue et al. 1997). Reductions in I_{to} may lead to the improper establishment of the plateau of the cardiac AP and to subsequent changes in Ca^{2+} ion release and loading that result from changes in L-type Ca^{2+} channels, $I_{Ca,L}$, and Na^+–Ca^{2+} exchange, effective mechanisms that increase the inotropic capability of the myocardium in the diseased state (Yue et al. 1997). Reduced I_K may also be responsible for improper plateau phase repolarisation of the AP with subsequent effects on the late phase of repolarisation mediated by inwardly rectifying K^+ (I_{Kir}) channels and hence prolongation of the AP and the QT interval of the ECG. Together these two effects may manifest themselves as early and/or late afterdepolarisations and the arrhythmias associated with them (Studenik et al. 2001).

Interestingly, Han et al. (2000) have explored K^+ channel heterogeneity and shown that canine Purkinje fibres express I_{to} currents that are functionally and pharmacologically different from ventricular I_{to} currents. Clinically, such heterogeneity also exists in I_{to} current.

The best characterised of the cardiac disorders involving a K^+ channel is the long QT syndrome (LQTS), the clinical effect that ushered in the discipline of SP over a decade ago. It is a specific cardiac disorder that is related to genetic alterations (either acquired or inherited) or occur due to drug blockade of the I_K channel. Regardless of the cause, it results in the development of a specific, distinguishable, cardiac phenotype called torsade de pointes or TdP. While a number of genetic loci (such as 11p15 in humans) have been identified that contain the genes responsible for LQTS, the majority of these loci encode α- and β-subunits for the I_K channel (Roberts and Brugada 2000).

In most mammalian species, a gene from the KCNQ (formerly K_vLQT) subfamily, KCNQ1, when co-expressed with the auxiliary subunit, minK (or IsK or KCNE1), combines to form functional K^+ channels that mimic the slowly activating component of the delayed rectifier current (I_{Ks}). LQTS1 is associated with abnormalities in both of these potassium channel subunits and is expressed as auditory and cardiac phenotypes in the Jervell and Lange-Nielsen syndrome (Casimiro et al. 2001). Using site-directed mutagenesis, Hoppe et al. (2001) have determined that mutations in the human *ether-a-go-go* (*Eag*) related gene (HERG) can suppress I_{Kr} and lead to significant transmural variability in beat-to-beat APD and ECG morphology. Also, KCNE1 mutagenesis can markedly suppress I_{Ks} and result in the development of early afterdepolarisation arrhythmias (EADs). Thus, delayed repolarisation predisposes the heart to EADs and provides a mechanism for

functional block resulting in the maintenance of a polymorphic re-entry pattern due to APD heterogeneity and potential for induction of TdP.

The association of HERG (Erg1 or KCNH2 gene) with minK (KCNE1) or KCNE2 (a minK homolog called MiRP) forms the molecular equivalent of the rapidly activating delayed rectifier K^+ channel (I_{Kr}) (Nerbonne 2000). Missense and frameshift mutations in a number of locations within the HERG α-subunit have been demonstrated and proposed as possible mediators of LQTS2; however, mutations in the KCNE2 β-subunit have been less numerous and have not been implicated in LQTS2 (Roberts and Brugada 2000). Mutations in the HERG sequences responsible for inactivation (both N- and C-type) and mutations within the pore loop alter the kinetics of I_{Kr} channels. These mutations, or equally they could be sites in which an NCE may interact, could potentially be associated with drug-induced LQTS2 and result in an increased tendency to development of cardiac arrhythmias.

The development of potent and selective methanesulfonamide drugs led to the discovery of an unexpected adverse event in the form of pronounced QT prolongation at low heart rates and induction of TdP. Subsequent studies indicated that the methanesulfonamide group of drugs were also potent blockers of native I_{Kr} channels in cardiac tissue as well as the molecular correlate of I_{Kr}, the HERG K^+ channels. Since they were potent blockers of these channels, they could essentially produce a chemical loss-of-function type of mutation. This uniform chemical loss-of-function type of mutation, if it occurs across a background of non-uniformly distributed K_V channels within the myocardium, may suggest a potential mechanism whereby these types of cardiac drugs (as well as the plethora of non-cardiac drugs that produce QT interval prolongation) produce a dispersion of refractoriness and a predisposition to TdP (Nalos et al. 2012). Drug-induced long QT syndrome developed into a major concern for the pharmaceutical industry, as well as global regulatory authorities since a wide range of non-cardiac marketed drugs (beginning with terfenadine) unexpectedly prolonged the QT interval precipitating TdP in patients. Terfenadine, a clinically used, non-sedating histamine (H_1) receptor antagonist, was the first drug to be shown to block HERG channels (Roy et al. 1996). Thus as a result of the effects of non-cardiac drug block of HERG ion channels and the involvement of these same channels in TdP arrhythmias and sudden cardiac death led to development of the regulatory requirements that appropriate safety studies be conducted in which this aspect of channel block and effects on the ECG be determined, the remit of SP.

7.3 The Inward Rectifier Potassium Current (I_{Kir})

The inward rectifier (K_{ir} or I_{K1}) K^+ channel can be described mathematically with a single gate that operates so fast (usually less than a ms) that g_{K1} can be treated as changing instantaneously with voltage; since channels close with depolarisation, there is in effect a *very* strong inward rectification. The inward rectification of these channels tends to hold the resting membrane potential close to V_K. These channels

are greatly dependent on K^+ and this probably accounts for the strong dependence of myocardial cell excitability on K^+.

The K_{ir} channel α-subunits have been defined and, like other K^+ channels, are widely distributed in the mammalian genome. More than 15 α-subunits have been defined and grouped into seven families ($K_{ir}1$–7). The $K_{ir}2$, $K_{ir}3$ and $K_{ir}6$ families appear to be the most important with respect to K^+ channel function in the mammalian myocardium. Within the $K_{ir}2$ family, the genes for subunits $K_{ir}2.1$, 2.2 and 2.4 combine to form I_{Kir} (Topert et al. 2000) while the $K_{ir}3.1$–3.4 subunits form G-protein-coupled inward rectifying K^+ channels (I_{KACh}) (Sakura et al. 1995). The ATP regulated inward rectifying potassium channels (I_{KATP}) are formed from α-subunits produced by genes for $K_{ir}6.1$ and 6.2 (Ashcroft and Gribble 2000).

7.3.1 Molecular Genetics of I_{Kir}

The K_{ir} channels exhibit the simplest structure of all of the K^+ channel families (see Fig. 5) described to date comprised of the most basic form of a K^+-selective pore and possibly the evolutionary starting point for K_V and K_{2P} channel formations (Nerbonne 2000). The pore-forming α-subunits exhibit two transmembrane-spanning sequences with a pore loop sequestered between them and intracellular N- and C-terminals. However, the sulfonylurea receptor (SUR) accessory subunits that combine with the α-subunits to form functional channels exhibit extracellular and intracellular N- and C-termini respectively and 17 transmembrane-spanning sequences in a 5-6-6 configuration (Ashcroft and Gribble 2000). Like the K_V and K_{2P} channels, functional K_{ir} channels are formed through the oligomeric association of α- and accessory subunits to form the K^+-selective pore-forming region. However, unlike these channels, some of the K_{ir} channels (such as I_{KATP} in pancreatic β-cells) only exist as functional channels in an octomeric form. The octomeric form results from the association of 4 K_{ir} subunits with 4 SUR subunits as opposed to typical functional tetramers. These functional K_{ir} channels then require homoligomerisation of subunits where 4 $K_{ir}6.2$ subunits and 4 SUR1 subunits co-assemble as opposed to hetero-oligomerisation (Ashcroft and Gribble 2000). The H5 loop of K_V channels and the signature sequence G(Y)G selectivity filter of the pore are found in all K_{ir} channels. Unlike the K_V channels, K_{ir} channels lack an intrinsic S4 voltage-sensing element and a mechanism for rapid N-type inactivation and therefore do not 'sense' and respond to changes in membrane potential in the manner of K_V channels. The K_{ir} channels are nonetheless affected by membrane voltage. The orientation of the M2 transmembrane-spanning sequences is believed to provide the permeability filter whereas M1–M2 orientation relative to each other is believed to provide the channel gating mechanism (Ashcroft and Gribble 2000). However, due to their early evolutionary appearance (e.g. as KcsA in some bacteria) and abundance in primitive intracellular structures (e.g. mitochondria), most K_{ir} channels play large roles in linking cellular metabolism to excitability.

7.3.2 Functional and Pharmacological Properties

Within the heart, I_{K1}, I_{KATP} and I_{KACh} play different roles due to their unique functional properties and channel distributions. I_{K1} is strongly active at potentials

close to the resting membrane potential; therefore, I_{K1} plays an important role in the maintenance of the AP plateau, rapid terminal repolarisation and resting membrane potential. Both I_{KATP} and I_{KACh} are ligand-gated currents and are therefore heavily regulated by both intracellular and extracellular messages. I_{KATP} is regulated by myocardial metabolic status and those elements that serve key functional roles in metabolism (e.g. glucose, ATP/ADP, CO_2 and pH) (Weiss and Venkatesh 1993). Therefore I_{KATP} will have a strong functional role in the regulation of cardiac excitability under both normal and abnormal conditions (e.g. ischaemia or infarction). I_{KACh} is sensitive to G-protein regulation in both a muscarinic (M) receptor dependent and independent manner and is therefore susceptible to modulation via the autonomic nervous system and by a variety of other G-protein-coupled receptor systems. As such, I_{KACh} is instrumental in vagal mediated heart rate modulation and beat-to-beat management during exercise and other physiological situations where rapid changes in heart rate occur.

Like most K^+ channels, the K_{ir} channels extrude intracellular K^+ in order to re-establish the membrane potential and to bring about cellular repolarisation in excitable myocardial cells. Therefore, activation of K_{ir} channels is expected to increase APD whereas a reduction of this current is expected to increase APD. An increase in I_{K1} is associated with a reduction in the J–T segment duration and a narrowing of the T-wave in the ECG. Wu et al. (1999) showed that I_{K1} blockade in the rabbit heart, with concomitant APD and QT_c prolongation, is associated with an almost complete flattening of the T-wave in accordance with the known relationship between T-wave amplitude and activation time. Interestingly, the same authors showed that I_{K1} blockade had a greater effect on T-wave morphology than did I_{to} blockade, thereby demonstrating the relative importance of the two K^+ channel types in rabbit ECG morphology. Additionally, blockade of I_{K1} with Ba^{2+} produced increases in the APD of both atrial and ventricular myocytes and prolonged the rate-corrected QT interval of isolated rabbit hearts without detrimental effects on conduction and no proarrhythmic potential (Wu et al. 1999). It was a result of these properties that drugs such as terikalant (RP58866) were investigated for their antiarrhythmic potential (Rees and Curtis 1995). Williams et al. (1999) have shown that I_{K1} is involved in the rate-dependent shortening of APD and that terikalant possesses positive rate-dependent prolongation of the AP, a unique profile compared to selective blockers of the I_K channel. Wu et al. (1999) have shown that terikalant maintains its ability to prolong APD at depolarised membrane potentials and abolish ischaemia-induced arrhythmias. However, terikalant also produces significant inactivation block of HERG and theoretically could predispose the heart to idiopathic LQTS and TdP arrhythmias. Farkas and Coker (2002) evaluated terikalant in a proarrhythmia model and showed that it produced limited induction (20 %) compared to clofilium (60 %). Due to the nature of I_{K1} activation and role in rate-dependent APD modification, drugs that selectively block I_{K1} were initially hoped to provide antiarrhythmic efficacy. Development of the class has halted.

The pharmacology of I_{KATP} channels has been very well studied. Briefly, sulfonylurea drugs such as glibenclamide bind with high affinity to the sulfonylurea receptor (SUR1) subunit and produce blockade of K_{ATP} channels. Diazoxide as well

as benzopyran derivatives such as cromakalim also bind to SUR1 but act to open K_{ATP} channels (Ashcroft and Gribble 2000). The role of I_{KATP} openers in the treatment of atrial and ventricular arrhythmic disorders is limited due to the drug's potential to reduce APD and thereby predispose cells to maintenance of re-entry circuits. However, in situations where bradycardia and much slowed conduction are associated with depressed myocardial function, the administration of I_{KATP} openers may be of use in reversing the depressive state. The role of I_{KATP} blockers on the other hand is much less clear. In some models I_{KATP} blockade by glibenclamide produces antiarrhythmic protection whereas in others the protection is either limited, not apparent and even detrimental (Barrett and Walker 1998). Like most of the K^+ channel pharmacology described in this chapter, the effects of I_{KATP} blockade by an NCE (or augmentation in myocardial disorders) will depend specifically on the role of the I_{KATP} channel in off-target drug activity and the selectivity of drugs with which I_{KATP} can be augmented in cardiac disorders. As a result of the strong link between I_{KATP} channels and cellular metabolism, these channels may be expected to have a limited, discernible contribution to the ECG under normal circumstances. However, these same channels might be expected to contribute significantly to observed changes in ECG morphology in diseased or metabolically compromised states.

The pharmacology of I_{KACh} is at least as diverse as the substantial pharmacology of G-proteins and the numerous receptors and intracellular signals that modulate these channels and will therefore not be discussed in detail here. I_{KACh} is highly expressed in atria and in particular the SA node. Less expression is found within the ventricles, in agreement with the fundamental role of this channel in autonomic regulation of heart rate and, therefore, may be significantly affected by pharmacological manipulation of atrial or SA node cells. The effects of I_{KACh} on APD and the ECG may be observed most readily through analysis of A-H and H-V cardiac electrograms since the channel may have very little influence on ventricular repolarisation.

Previously we described the regulation and function of K_V channels in AF (see K_V section above) and the apparent paradox that exists whereby a reduction in K_V channel density occurs with a reduced APD in AF following remodelling in the heart. This physiological effect is similarly relevant to consideration of drug effects in the ventricle. The I_{K1} channel may also play a role in APD reduction during AF-induced remodelling in addition to the effects of a reduced I_{Ca} and in keeping with its strong function in rate-dependent APD shortening (Farkas and Coker 2002). Bosch et al. (1999) have demonstrated that increases in both I_{K1} and I_{KACh} densities in AF remodelled human atria, along with reduced I_{Ca}, may circumvent the protective reductions in K_V currents exhibited. Therefore, selective pharmacological manipulation of I_{K1} and/or I_{KACh} with drugs such as adenosine is useful in the treatment of supraventricular arrhythmias such as AF.

8 The Electrocardiogram

Electrical activity in electrically excitable cells results from the opening and closing of ion channels in a voltage- and time-dependent manner. The depolarisation of a single cardiac cell results in the electronic spread of electrical activity to adjacent cells and the production of current which flows in the direction of depolarisation. A second, repolarising current, is established in order to restore electrical excitability to cells. If these currents are recorded in individual cells we observe an AP, if they are recorded on the surface of the body we observe an electrocardiogram (ECG).

Thus, the electrocardiogram (ECG) is most easily defined as the global summation of all the electrical activity that is generated by cells within the various regions of the heart. It represents the rate of change of voltage across all the cell membranes as a function of time, $\Delta V_m/dt$. Willem Einthoven first developed the ECG in 1903 (Einthoven 1912). It was realised early on in its development that components of the electrical signal coincide with components of electrical signals that arise within cardiac muscle. These ECG intervals represent activation and inactivation properties of the myocardial cells.

The intervals that are defined by the ECG present both the clinician and safety pharmacologist with a fundamental tool with which to diagnose disease or investigate drug safety and elucidate mechanisms responsible for ECG changes that could suggest development of aberrant electrical activity (i.e. arrhythmia) within the heart. The ECG is a sensitive enough diagnostic tool to delineate drug-induced changes in electrical activity or diseases such as regional myocardial infarction or cardiac hypertrophy or can provide indications of abnormal electrolyte levels.

Within conducting tissue, there exists an important cellular hierarchy which ensures proper genesis and conduction of current and hence contraction of cardiac muscle since all cells in the heart are capable of generating electrical impulses spontaneously. Thus, under some conditions, various cells in the heart can display the property of automaticity. The sino-atrial node (SAN) dominates and is the primary pacemaker of the heart, as it possesses the highest rate of automaticity compared to any other cell in the heart. Electrical impulses generated by cells within the SAN are conducted across atrial conduction pathways, through the AV node (AVN) into the bundle of His. The impulse then moves down the left and right bundle branches into Purkinje fibres that are diffusely spread throughout the myocardium and terminate in muscle cells. The propagation of an electrical impulse generated in the SAN through cardiac tissue is proportional to the volume of tissue that is electrically excitable. Hence, the various deflections observed in the ECG waveform also reflect the changing size of the tissue that is generating and conducting the current.

Electrical activity generated by atrial depolarisation is recorded on the ECG as the P-wave. Atrial repolarisation occurs immediately, as the ventricles depolarise; however, since the ventricular depolarisation occurs at the same time, the repolarisation wave for the P-wave of the atria is not observed on the ECG. Rather, a large QRS complex is observed to follow the P-wave. Ventricular depolarisation is a composite of the Q, R and S waves. The Q-wave represents depolarisation of the

interventricular septum, while the R-S complex represents the simultaneous depolarisation of both the left and right ventricles. Due to the dissimilarity in muscle mass between the ventricles, the QRS complex, by convention, represents depolarisation of the left ventricle. Repolarisation of the ventricles is recorded on the ECG as the T-wave. Occasionally a U-wave is present in the ECG (as slow heart rates) which represents the final stages of ventricular repolarisation. It usually results from repolarisation of papillary muscles or ventricular septum and occurs after most of the ventricular tissue has repolarised. In addition to waves and complexes, the ECG contains intervals and segments that represent flat or isoelectric sections on the ECG record.

The PR interval represents the time required for electrical impulse conduction from its origin at the onset of atrial depolarisation through the atrial conduction system to the ventricular myocardium. It generally represents conduction time across the AVN. Changes in this interval, in most species, reflect changes in Ca^{2+} channel function; however, in the rat Na^{+} channels are dominant (Botting et al. 1985). Unlike the PR interval, the QT interval represents the ventricular refractory period and includes depolarisation and repolarisation of ventricular muscle. In contrast to the atria, the AP in ventricular tissue is long (≈300 ms), which is a time interval that is similar to the duration of the QT interval. Thus, the QT interval is an approximate measure of ventricular repolarisation and thus K^{+} channel function.

Only three segments of the ECG will be discussed. The PR segment represents the time required for an electrical impulse to propagate from the AVN through to the ventricular myocardium. It reflects the time between the end of atrial depolarisation and the onset of ventricular depolarisation. The ST segment is one of the most important measures of the ECG since it represents the early phase of ventricular repolarisation. If a depression in this segment of the ECG occurs, it can be used to diagnose conditions whereby a reduction in coronary blood flow occurs to the heart. An elevation in this segment occurs in a damaged area to the ventricular wall that may be associated with myocardial ischaemia or infarction. However, any abnormality in these measures may be indicative of some underlying pathophysiological process that alters the AP in cardiac cells that are a consequence of changes in voltage-gated ion channel(s) in tissue. Note that if the ST segment of the ECG is absent, the T-wave is thought of as beginning at the end of the QRS complex, or the J-point. Most often a J–T interval can be measured as the distance from the convergence of the J-point and the isoelectric baseline to the end of the T-wave (i.e. where the T-wave returns to the isoelectric line). However, sometimes the onset and end of the T-wave are difficult to determine. This measure provides a representation of ventricular repolarisation independent of the QT interval and is sometimes used as an indirect assessment of the heterogeneity of repolarisation.

Since the ECG is a composite of many voltage-gated ion channels, it may not be consistent between various animal species. The variability may be quantitative, i.e. different currents or current densities in the hearts of different species may result in differences in the duration of various segments or intervals. The variability may also be qualitative, i.e. the ECG may exhibit a different shape as a result of the

expression of an ion channel distinct from that found in the human heart or in other species.

These marked variations can be observed in the ECGs from animals and are especially noticeable when compared to ECGs recorded in humans. While little disparity exists regarding the role of both Na^+ and Ca^{2+} channels in the hearts of various species, the rat being an exception, important species and regional differences exist in the contribution K^+ channels make to repolarisation of the cardiac AP. Studies have shown that electrophysiological distinctions can be made between epi- and endocardial tissue in many species including the dog (Yan et al. 1998). In canine ventricles, epicardial, mid-myocardial (M-cells) and endocardial cells display distinct electrical properties and hence different AP morphology (Antzelevitch 2004). Wang et al. (1998) have characterised epicardial and endocardial differences in APD in human atrial tissue and suggest that the ionic mechanism for these differences is due to different amplitudes of the transient outward K^+ current (I_{to}). Similar differences are also seen in ventricular tissue. In the rat the density of the fast component of I_{to}, $I_{to,f}$, current is greater in the atria than in the ventricle while in the mouse the opposite is observed (Nerbonne 2000). However, this current is ubiquitous in the hearts of most species including humans, rat, mice, feline, canine and ferrets. Not unlike $I_{to,f}$, other K^+ current densities vary in myocardial cells. The delayed rectifier (I_K) current is greater in the atria than in the ventricles of the guinea pig (Sanguinetti and Jurkiewicz 1991). Thus, the disparity in level of K^+ channel expression and the resulting current density contribute significantly to the variations in AP waveforms observed in myocardial cells in the atria and ventricles and in turn result in differences in the recorded ECG.

The diversity of ion channels present in cardiac tissue has been observed, to a greater degree than by simply recording currents from whole and isolated cardiac tissue, through molecular cloning procedures. The molecular cloning of various ion channels, their component current subtypes and accessory subunits has resulted in the establishment of an even greater level of ion channel diversity than could have originally been anticipated. The complexity in ion channels arises from the diverse protein nature of the individual pore-forming α-subunits present in cardiac tissue and also as a result of alternative splicing of transcripts for these proteins. The large number of accessory subunits specific for the ion channel again complicates matters.

8.1 ECG Changes that Can Occur in the Myocardium

The ECG can be used as a means to detect cardiac related adverse drug effects or as a diagnostic aid for disease since many of its intervals and segments are sensitive to electrical, biochemical and pathological changes in myocytes. In humans, common causes of arrhythmias include myocardial ischaemia, myocardial infarction or reperfusion of a previously ischaemic myocardium. These conditions can be readily reproduced in both intact and isolated hearts in many species. While the pathology of arrhythmias may not appear to be relevant to the safety pharmacologist, the

mechanism(s) derived from arrhythmia studies that explain arrhythmogenesis have direct implications in the development of the CIPA paradigm and its ability to assess the 'proarrhythmia potential' for a new drug. The mechanism(s) developed will be important in the interpretation of ion channel binding data and predicted changes in the AP using in silico models. Thus, a review of the pathological changes associated with the ischaemic myocardium is warranted since an understanding of such changes is likely to better arm the safety pharmacologist when drug-mediated adverse cardiac effects produce altered depolarisation (PR and QRS interval) and repolarisation (QT or T-wave morphology) effects.

The hearts of many non-clinical species used in SP studies including primates, pigs and rats do not have extensive coronary collaterals, i.e. these coronary arteries are end-arteries, like humans (Schaper et al. 1986). Thus, when a coronary artery is occluded an area is rendered uniformly ischaemic. If occlusion of an artery persists, irreversible damage occurs and progresses until infarction results. The time dependency of the onset of arrhythmias after occlusion is characteristic for many species. For example, arrhythmias first occur in conscious chronically prepared rats 5–15 min after occlusion during the reversible stage of ischaemia (Clements-Jewery et al. 2007). The most common arrhythmias seen include premature ventricular contractions (PVC), ventricular tachycardia (VT) and ventricular fibrillation (VF). A second arrhythmic period occurs 1–3 h after occlusion during the evolution of the infarct and consists of PVC, VT and VF (Clements-Jewery et al. 2007). Various factors influence the severity and incidence of arrhythmic outcomes after occlusion. These include both the size of the ischaemic zone and the serum K^+ concentration.

Well-defined ECG changes commonly occur in many animal models of coronary artery occlusion. These time-dependent changes delineate the course of events associated with ischaemia. Typically, occlusion of a coronary artery results in a change in the R-wave amplitude that begins within minutes after occlusion followed by an elevation in the S–T segment and many types of arrhythmias including VT and VF. The mechanism by which S–T segment elevation occurs is not known but is suggested to relate to changes in cellular biochemistry and electrophysiology (possibly due to ion channels' up- or downregulation) as they relate to anoxia and deranged energetics. ECG responses to ischaemia are well defined in many animal species.

Ischaemia also produces changes in the extracellular milieu of the cell. Case et al. (1979) showed that ischaemia increases extracellular K^+ and is accompanied by changes in pH, O_2 and CO_2 levels within the ischaemic zone. These changes in ion concentrations have become better defined with improved experimental techniques including ion-sensitive electrodes, nuclear magnetic resonance (NMR) and voltage-sensitive dyes.

Briefly, the intracellular events that occur as a result of ischaemia includes a reduction in pH, an elevation in Na^+ due to partial suppression of the Na^+/K^+-ATPase pump and an increase in Ca^{2+}. Many of these changes are not homogeneous within the ischaemic myocardium due to local variations in accumulation and diffusion of cellular wastes such as CO_2. This may have implications in the changes observed in the ECG since ion channel properties change as well as in the

development of arrhythmogenic circuits (see below). Accompanying the intracellular changes are extracellular changes. Within the ischaemic myocardium, there is a triphasic increase in K^+ which, unless reversed, results in an irreversible loss in membrane integrity (Hill and Gettes 1980). A reduction in extracellular pH occurs that parallels the change in extracellular K^+ levels. In a similar manner, intracellular events are varied and contribute to the heterogeneity within ischaemic tissue. In addition to the local micro-inhomogeneities of ions which occur within the myocardial extracellular space, Hill and Gettes (1980) also showed that a disparity exists between the centre and the border zone of the developing ischaemic tissue and still yet between the myocardial subepicardium and subendocardium. These transmural differences have been associated with the wave-like spread of ischaemia from the endocardium to the epicardium.

Abnormal impulse generation can arise from oscillations in the membrane potential and has been characterised as triggered rhythms (Binah and Rosen 1992). These triggered rhythms occur in two forms: early or late afterdepolarisations (EADs or DADs).

8.2 Early Afterdepolarisations

EADs interrupt either Phase 2 or 3 repolarisation of the AP. If these afterdepolarisations attain sufficient thresholds, they may produce triggered responses and induce single or multiple extrasystoles and even VT. The EAD is an oscillatory potential that is sensitive to frequency and often occurs at slow stimulation rates. EAD activity has been shown in vitro using many types of isolated cardiac muscle and various cell types including mid-myocardial cells (M-cell) (Antzelevitch 2004). Induction of EAD activity can be induced by a variety of drugs that block sodium and potassium channels as well as by catecholamines.

The ionic basis for EAD development is unclear. However, studies suggest the involvement of the slow inward Ca^{2+} current (I_{si}) of the cardiac L-type Ca^{2+} channel during the plateau of the AP. Essentially, I_{si} re-activation acts as a depolarising charge carrier during the depolarising phase of the EAD. Prolongation of the plateau phase of the AP allows for an increased time for Ca^{2+} channel recovery which enhances the inward current, thereby depolarising the membrane and sustaining the EAD. Other proposed mechanisms include a reduction of outward K^+ currents resulting in slow repolarisation and an increase in Na^+ window current associated with a prolonged plateau of the AP. Ultimately, arrhythmias which result include the long Q–T syndrome and TdP; however, the genesis and maintenance of these arrhythmias by an EAD mechanism remain unclear.

8.3 Delayed Afterdepolarisations (DADs)

Transient depolarisations that occur during Phase 4 of the cardiac AP are dependent upon the rate of the preceding AP (Antzelevitch and Sicouri 1994). Unlike EADs

the amplitudes of DADs increase with decreasing cycle lengths. DADs have been observed under a variety of experimental conditions all of which have a similar end result, i.e. intracellular Ca^{2+} overload. High intracellular Ca^{2+} concentrations saturate sarcoplasmic reticulum sequestration mechanisms resulting in Ca^{2+} oscillations due to Ca^{2+}-induced Ca^{2+} release (Clusin 2003). The ionic currents that contribute to this mechanism are not known. Ischaemia, digitalis and catecholamines can directly produce DAD by enhancing Ca^{2+} entry into cells. Thus, Ca^{2+} channel blockers, such as verapamil, abolish DADs. Studies show that Na^{+} channel blockers including lidocaine and the K^{+} channel activator, pinacidil, may all effectively suppress DAD and DAD-induced triggered activity (Clusin 2003).

8.4 Re-entrant Arrhythmia Pathways

The major cause of ventricular arrhythmias is due to re-entry. Re-entry has been subdivided into either circus-movement excitation or reflection (Wit and Rosen 1983). The basic model for re-entrant circus movement is a bifurcating Purkinje fibre bundle attached to the ventricle that gives rise to different anatomical conduction pathways. Re-entry occurs when antegrade conduction of the impulse is extinguished at a site of unidirectional block. This type of block may arise from ischaemic damage of previously normal conduction pathways. If normal conduction continues in the other branches of the pathway, an impulse can retrogradely enter the area of unidirectional block where its conduction is slowed but not extinguished. The impulse can then emerge from this depressed area and, providing that the cells are not refractory, re-excite the tissue proximal to the area of block. This generates premature ventricular complexes (PVC) which can remain as such, or deteriorate into VT or VF along a pre-existing pathway and usually results in VT. Random re-entry of impulse propagation results when electrophysiological differences exist between areas of cardiac muscle. The development of ischaemia in cardiac muscle is a dynamic process; therefore, the pathway is not constant for the impulse which circulates. It may fractionate, produce multiple re-entrant circuits and result in VF.

The mechanisms suggested for arrhythmogenesis are complex and all, under ischaemic conditions, may play a significant role. It is most likely that re-entry dominates during VT and VF while the mechanisms for PVCs are less clear. However, the changes observed in the ECG under these conditions provide a reliable index of myocardial ischaemia; further studies in the area of ion channel physiology, molecular biology and how ion channels change during conditions of ischaemia may provide us the fundamentals for novel therapeutic drug development.

9 Conclusion

Thus, SP continues to seek to provide validation and refine methods for use in preclinical hazard detection of NCE adverse effect liability. It does so in accordance with the scientific methods that rapidly progress with technology advances for functional measures. Additionally, while SP seeks to organise the strategy of implementation of methods according to regulatory guidance documents issued by the ICH and regulatory authorities, it aims to maximise translational value of the data acquired in hope of better predictivity in drug development. Currently there is an evaluation ongoing that thinks 'outside the box' with regard to characterising the safety profile of novel drugs in development. The CIPA initiative seeks to provide a greater comprehensive assessment of the direct proarrhythmic potential of a drug. It is hoped that implementation of this assay which focuses on the evaluation of multiple cardiac ion channels can be ultimately developed to assess drug effects on human cardiac electrophysiology with perhaps induced pluripotent stem cells acting as the validated, sensitive and specific cellular assay. Implicit understanding is required of basic ion channel biophysics and cardiac electrophysiology of sodium, calcium and potassium channels that constitute the AP and arrhythmia mechanisms. Thus, the paradigm shift related to CIPA is being realised by safety pharmacologists and because of this the discipline remains in evolution and this coverage of new aspects of safety is certain to expand to provide better, or modified, guidance in the next few years.

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Sensitivity and Specificity of the In Vitro Guinea Pig Papillary Muscle Action Potential Duration for the Assessment of Drug-Induced Torsades De Pointes Liability in Humans

Joffrey Ducroq

Contents

Abstract

The ICH S7B document, which provides guidance for the preclinical cardiovascular evaluation of pharmaceutical new chemical entities (NCE), is essentially focused on drug-induced QT lengthening, a biomarker for the proarrhythmic adverse drug reaction, torsades de pointes (TdP). In 2005, this guidance recommended the IKr assay and the in vivo QT telemetry study as mandatory assays for detecting potential torsades de pointes liability and relegated the cardiac action potential (AP) assay as a follow-up study. The IKr assay has become a mandatory screening tool in the early development and safety assessment process. Using only the IKr assay as a go/no go decision arbiter is regrettable since, due to the low specificity of the model (positives that are false for proarrhythmia liability, e.g. verapamil), promising, safe NCEs may be inadvertently discarded. Inclusion of additional medium throughput assays should be performed early to confirm or balance the putatively unfavourable IKr result with positive discovery model output (Pugsley et al., J Pharmacol Toxicol Methods 60:24–27, 2009). In the present chapter, the predictive value of in vitro

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guinea pig papillary muscle action potential assay will be discussed in terms of sensitivity and specificity and compared to currently available preclinical models such as IKr/hERG assay, dog Purkinje fibre action potential and in vivo QT measurements in dog and cynomolgus monkey.

Keywords
Action potential • Guinea pig • Papillary muscle • QT PRODACT • QT prolongation • Sensitivity • Specificity • TdP

1 Introduction to Preclinical Cardiac Safety Pharmacology

In the last few decades, many drugs such as the gastrointestinal prokinetic agent cisapride and the non-sedating antihistamines astemizole and terfenadine have been withdrawn from the market because of their propensity to induce torsades de pointes (TdP), a potentially life-threatening tachyarrhythmia (Redfern et al. 2003). TdP has been shown to occur in association with prolongation of the QT interval of the electrocardiogram, which is indicative of ventricular AP repolarisation delay (Viskin 1999). Moreover, the vast majority of torsadogenic compounds were found to block the hERG-encoded current (IKr), which is one of the principal repolarising currents in human ventricular cells (Sanguinetti and Jurkiewicz 1990). The likelihood of TdP, when a drug prolongs the QT interval, is nevertheless very low, estimated in the range of 1 in 2,000–20,000 patients, depending in part on other risk factors in the treated population (Bass et al. 2005) but largely determined by stochastic factors. Therefore, it is difficult to evaluate the torsadogenic liability of NCEs during early phase I clinical trials by determining TdP incidence or even by measuring QT prolongation—the former being perhaps too infrequent to detect in a feasible study sample and the latter being a surrogate biomarker of limited value (Pugsley et al. 2009). For this reason, the choice of an adequate strategy in preclinical cardiac safety pharmacology studies has become a major subject of concern shared by both the pharmaceutical industry and global regulatory authorities in the drug development process (Pugsley et al. 2008).

With regard to this matter, in 2005, the International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use has published a final version of the ICHS7B guidance (CHMP/ICH/423/02), in order to ‘describe a non-clinical testing strategy for assessing the liability of a test substance to delay ventricular repolarization’. Clearly repolarisation delay is merely a biomarker for TdP, but this is the guidance nevertheless. In this guidance, the in vitro IKr assay and the in vivo QT assay are suggested models to be used in a general non-clinical testing strategy. In the guidance, the in vitro AP assay has been removed from the recommended core set of studies and relegated to ‘optional’ or ‘follow-up’ test status, giving the

impression of a model providing little value to safety pharmacology integrated risk assessment strategy.

Limiting the in vitro cardiac preclinical investigations to only the IKr assay for the selection of a lead candidate before regulatory-predicated in vivo QT studies in dogs or non-human primates (NHP) assay would be a serious strategic mistake for two main reasons. First, the predictive value of preclinical models to TdP liability in humans is commonly discussed in terms of 'sensitivity' and 'specificity'. Sensitivity is defined, from a panel of compounds, as the number of true positives (positive for both the preclinical assay and the clinical QT study) divided by the sum of true positives and false negatives (negative for preclinical assay but positive for clinical QT study). Specificity is defined as the number of true negatives (negatives for both preclinical assay and clinical QT study) divided by the sum of true negatives and false positives (positive for preclinical assay but negative for clinical QT studies). It was clearly demonstrated that if the IKr assay shows good sensitivity, the specificity of the model is likely to be poor, especially when high drug concentrations are tested, i.e. 10- to 30-fold the free clinical plasma concentration (Wallis 2010). This result is all the more important given that a safety margin of 30-fold the expected maximal therapeutic plasma concentration (C_{max}) is recommended for assigning an NCE as having a potential TdP liability (Redfern et al. 2003). In the literature, the most cited example of a false positive compound in the IKr assay is verapamil, a class IV antiarrhythmic drug which, in addition to its primary desired therapeutic calcium channel blocking property, also potently reduces IKr (with an IC_{50} of 100–800 nM). Despite its high affinity for IKr, verapamil is devoid of QT-prolonging action in human hearts even though the total and free effective therapeutic plasma concentrations are 250–810 nM and 25–81 nM, respectively (Omata et al. 2005). The 'neutral' effect of verapamil on QT is explained by the fact that the expected AP lengthening induced by IKr inhibition is counterbalanced by the AP-shortening effect associated with blockade of L-type calcium channels. Secondly, cardiac safety pharmacology studies should never be limited to IKr-related QT prolongation but should accommodate the fact that TdP is associated with modulation of other ionic currents mediated by other channels such as the slow component of the delayed rectifier current (I_{Ks}), the persistent sodium current (I_{NaL}), the inward rectifying potassium current (IK1) and the transient outward potassium current (I_{to}). Moreover other types of severe arrhythmias may be associated with modulation of I_{Na} and I_{CaL}. The AP prolongation assay, performed using isolated papillary muscle or Purkinje fibre, constitutes a more integrative readout than the IKr assay, since all ventricular ion channels are involved.

In this chapter, we will focus on the papillary muscle action potential assay and compare the predictive value of this model in terms of sensitivity and specificity to that of the IKr assay, the canine Purkinje fibre action potential and the in vivo dog and monkey QT.

2 The Guinea Pig Isolated Papillary Muscle Action Potential Assay

In safety pharmacology studies, the papillary muscle is mainly used for the evaluation of the effects of NCEs on AP parameters. Indeed, the use of the poorly specific IKr assay as a unique in vitro screening tool before conduct of in vivo studies increases the risk of discontinuing development of promising, safe and effective compounds during the selection of a lead candidate. Taking this matter in consideration, pharmaceutical companies such as Boehringer Ingelheim decided to integrate, systematically, the guinea pig papillary muscle AP assay as a complementary tool to confirm or attempt to balance the IKr assay result (Guth et al. 2004). In case of any discrepancy between the two in vitro assays, the in vivo dog telemetry study, recommended as a core battery study by the ICH S7B guidance, arbitrates between any opposite results.

2.1 Why in Guinea Pig?

Papillary muscle APs are mainly recorded from cardiac tissue derived from either guinea pig or rabbit with a preference for guinea pig for primarily two technical reasons. In 'large' papillary muscles, the middle of the muscle may have a reduced access to the oxygenated perfusion medium. Therefore, the size of the papillary muscle should be as small as possible to avoid the risk of recording from potentially ischemic tissue that is likely to occur in large species such as the dog and larger non-human primates (Guth 2007). Moreover, the papillary muscle is a contractile tissue and contractile movements of the muscle make stable placement of the microelectrode in a cell challenging. It may be most desirable to the safety pharmacologist to ensure that the papillary muscle be small and consistent in size from preparation to preparation in order to reduce the potential for a risk of loss of microelectrode impalement during the experiment. A second impalement may be possible, albeit not optimal, when it has been quickly obtained and the experimenter ensures that the AP parameters have not significantly changed between the two impalements. However, it is generally recommended that such preparations be discarded from all experiments in which impalement is lost because it is not possible to record all events that transpired during the time lapse.

2.2 Specificity and Sensitivity of the Guinea Pig Papillary Muscle Action Potential Assay: The QT PRODACT Initiative

A project named 'QT Interval Prolongation: Project for Database Construction (QT PRODACT)' was organised by pharmaceutical companies belonging to the Japan Pharmaceutical Manufacturers Association (JPMA) and contract laboratories belonging to the Japan Association of Contract Laboratories for Safety Evaluation (JACL). The aim of this project was first to construct a non-clinical database to

evaluate predictive values of three preclinical models: hERG/IKr, guinea pig papillary muscle action potential and in vivo dog QT measurements for the assessment of QT prolongation and TdP liability in humans. The database used 11 positive reference compounds (astemizole, bepridil, cisapride, disopyramide, E-4031, haloperidol, MK-499, pimozide, quinidine, terfenadine and thioridazine) and 10 negative reference compounds (amoxicillin, aspirin, captopril, ciprofloxacin, diphenhydramine, flecainide, lidocaine, nifedipine, propranolol, verapamil) where reference drug classification was based on clinical QT prolongation data and evidence of TdP occurrence (Hayashi et al. 2005). The QT PRODACT results were published in a special issue of the *Journal of Pharmacological Science*. To our knowledge, if the predictive values of IKr, dog Purkinje fibre and in vivo dog QT assays have been evaluated later by other cooperating groups such as it was done in the ILSI-HESI initiative, only QT PRODACT initiative evaluated the sensitivity and specificity of the papillary muscle action potential assay.

The first paper of the *Journal of Pharmacological Science* special issue focused on the guinea pig papillary muscle action potential assay (Hayashi et al. 2005). In this study, the authors used two action potential parameters as predictive markers: APD_{90} and $APD_{30–90}$. All compounds were tested at three pacing frequencies (0.5, 1 and 2.5 Hz). Three or four concentrations were tested for each compound, based for the positive compounds on preliminary results or previous studies showing the maximum effects on action potential prolongation and those of the negative compounds on the maximum soluble concentration or set at a supra-therapeutic plasma clinical concentration. For one parameter (APD_{90} and $APD_{30–90}$), the authors considered a result as positive if, for at least one tested concentration at 1 Hz, the statistical analysis showed a statistically $p < 0.05$ and/or a 10 % increase vs. baseline values. Table 1 shows sensitivity and specificity of the assay using APD_{90} or $APD_{30–90}$ as biomarkers for TdP liability for the three tested concentrations using these discriminating criteria, not only for 1 Hz pacing frequency but also for 0.5 and 2.5 Hz. We can first conclude that the modulation of pacing rate did not really influence the sensitivity and the specificity of the assay. Hayashi et al. (2005)

Table 1 Sensitivity and specificity of the guinea pig papillary muscle action potential assay for QT and TdP liability using APD_{90} or $APD_{30–90}$ as predictive biomarkers. Predictive values were calculated for three tested pacing frequencies independently from the tested concentrations for all compounds. An in vitro result is considered positive for a compound if at least one concentration showed a statistically significant *and/or* ≥ 10 % increase in the selected parameter (APD_{90} or $APD_{30–90}$) vs. baseline values

	0.5 Hz		1 Hz		2.5 Hz	
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
APD_{90}	0.82 (9/11)	0.70 (7/10)	0.82 (9/11)	0.80 (8/10)	0.82 (9/11)	0.80 (8/10)
$APD_{30–90}$	0.91 (10/11)	0.50 (5/10)	0.91 (10/11)	0.50 (5/10)	0.91 (10/11)	0.60 (6/10)

Sensitivity is defined as the number of true positives divided by the number of clinical positive compounds and specificity as the number of true negative compounds divided by the number of negative clinical compounds

Table is based on data presented in the *supplementary materials* of the Hayashi et al. study (2005)

concluded that APD_{90} showed a moderate sensitivity of 0.82 because two positive clinical compounds (pimozide and terfenadine) were not detected but also showed a relatively good specificity of 0.80 (two negative clinical compounds, ciprofloxacin and flecainide increased APD_{90}). When $APD_{30–90}$ was considered, the sensitivity increased to 0.91 for all pacing rates since pimozide became positive but, on the other hand, the number of false positives strongly increased. Indeed, specificity fell to 0.50 since half of the negative compounds increased $APD_{30–90}$ at 0.5 and 1 Hz. It is interesting to note that, in the ILSI-HESI cardiovascular subcommittee initiative study, the two false negative compounds for APD_{90} (terfenadine and pimozide) were also false positive in the dog Purkinje fibre preparation using the same parameter and that pimozide also became positive using action potential triangulation as a predictive marker (Hanson et al. 2006).

Looking at detailed results of the Hayashi et al. (2005) study in the supplementary materials, we can see that the negative clinical compound ciprofloxacin was considered positive for APD_{90} in papillary muscle by the authors even if the observed significant increase was only 3 %. Instead of using a $p < 0.05$ *or* ≥ 10 % criteria to discriminate negative and positive in vitro results as was done by Hayashi et al., considering a $p < 0.05$ *and* ≥ 10 % criteria would convert ciprofloxacin from false positive to true negative. However, bepridil would in turn not have been detected as a positive compound.

It is interesting to observe what the results of the Hayashi et al. study (2005) would have been if the authors have used a lower but statistically significant threshold ($p < 0.05$ *and* ≥ 7 %) discriminating criteria for all pacing frequencies (Table 2). If the predictive value is not globally improved for APD_{90}, we can see that considering $APD_{30–90}$ and focusing on the frequency of 2.5 Hz, it would keep high the good sensitivity of $APD_{30–90}$ (0.91), but it would also increase its specificity from 0.60 to 0.80 (a value considered as good by Hayashi et al. for APD_{90} in the original article). Thus, changing the criteria and analysed frequency may improve the predictive value of the guinea pig action potential assay to predict TdP liability in humans.

Table 2 Sensitivity and specificity of the guinea pig papillary muscle action potential assay for QT and TdP liability using APD_{90} or $APD_{30–90}$ as predictive biomarkers. Predictive values were calculated for three tested pacing frequencies independently from the tested concentrations for all compounds. An in vitro result is considered positive for a compound if at least one concentration showed a statistically significant *and* ≥ 7 % increase in the selected parameter (APD_{90} or $APD_{30–90}$) vs. baseline values

	0.5 Hz		1 Hz		2.5 Hz	
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
APD_{90}	0.64 (7/11)	1.0 (10/10)	0.64 (7/11)	1.0 (10/10)	0.82 (9/11)	0.90 (9/10)
$APD_{30–90}$	0.91 (10/11)	0.60 (6/10)	0.91 (10/11)	0.60 (6/10)	0.91 (10/11)	0.80 (8/10)

Sensitivity is defined as the number of true positives divided by the number of clinical positive compounds and specificity as the number of true negative compounds divided by the number of negative clinical compounds

Table is based on data presented in the supplementary materials of the Hayashi et al. study (2005)

If the article of Hayashi et al. constitutes the first important work on guinea pig papillary muscle allowing to give a ruling on the assay in terms of sensitivity and specificity, the QT PRODACT initiative not only focused on this in vitro model but also evaluated the same set of compounds in vivo on QTc interval in anaesthetised dog, conscious dog and conscious cynomolgus monkey. It was therefore essential to evaluate the predictive value of the guinea pig papillary muscle action potential in comparison with other evaluated models. This analytical work was performed by Omata et al. (2005). The authors compared in vitro papillary muscle results with in vivo dog and monkey data and published IKr/hERG results for the 21 compounds of the database. First, the authors calculated the EC_{10} for APD_{90} and $APD_{30–90}$ in guinea pig papillary muscle action potential (i.e. a concentration inducing a 10 % increase in the parameter), the EC_{10} [unbound] value for in vivo QTc in dogs and monkeys (plasmatic unbound/free plasma concentration that produced a 10 % increase in QTc) and also the IC_{50} (half maximal inhibitory concentration) for the IKr/hERG assay. Then, they compared EC_{10} values of papillary muscle, in vivo dog and monkey and IC_{50} for hERG assay to determine which model would show the lowest values for the set of clinical positive compounds and, as a result, reveal the most sensitive assay to detect clinical TdP liability. As expected from the work of Hayashi et al. in guinea pig papillary muscle, EC_{10} values for $APD_{30–90}$ were lower than those of APD_{90} for all positive control torsadogenic compounds. Nevertheless, published IC_{50} values for IKr were lower than guinea pig $APD_{30–90}$ EC_{10} for most all positive compounds (with the exception of E-4031 and MK-499 for which values were similar), and there was no clear correlation between EC_{10} for $APD_{30–90}$ and IC_{50} for IKr. In the same way, EC_{10} values for both guinea pig APD_{90} and $APD_{30–90}$ were lower than EC_{10} [unbound] for most compounds. Only weak correlation was observed between the in vitro EC_{10} for $APD_{30–90}$ and dog EC_{10} [unbound], and no correlation was observed between the in vitro EC_{10} for APD_{90} and dog EC_{10} [unbound]. Since EC_{10} [unbound] values for conscious monkeys were similar to those of both anaesthetised and conscious dog, the authors concluded that the hERG assay and the in vivo QT assay in dog and monkey are able to detect positive compounds, i.e. 'hits', at lower concentrations than the guinea pig papillary muscle assay; however, the safety pharmacologist should note that it presumably depends on the ratio of therapeutic drug concentration to potential torsadogenic concentration in vivo that matters. Not only did the dog and monkey QT assay detect clinical QT-prolonging drugs at lower concentration than guinea pig papillary muscle assay, these in vivo QT assays also did not normally show false positive results since no negative compounds prolonged QTc by at least 10 % in the QT PRODACT study with the exception of nifedipine in conscious dogs. In vivo QT can then be considered at least as good as guinea pig papillary muscle in terms of specificity.

As stated above in the introduction, Redfern et al. (2003) have shown that a safety margin of 30-fold the unbound therapeutic plasma concentration in man (free effective therapeutic plasma concentration, free ETPC) should be taken into account when evaluating an NCE. Consequently the sensitivity and specificity of preclinical assays are commonly compared at fixed clinical exposure multiples.

Table 3 Sensitivity and specificity of non-clinical QT assays to predict the outcome of a clinical QT study. A preclinical result is considered positive for a compound if, at a preclinical concentration exposure (1-, 10- or 30-fold the unbound drug concentrations achieved in the clinic), a minimum 10 % increase is observed (hERG and canine Purkinje fibre assays) or a minimum 10 ms increase in QT is recorded (in vivo dog) vs. baseline values

Clinical exposure multiple	hERG		Canine Purkinje fibre		In vivo dog	
	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
2	0.82	0.75	0.2	1.0	0.83	0.86
10	0.90	0.38	0.33	0.80	0.83	0.33
30	0.90	0.14	1.0	0.40	0.83	–

Sensitivity is defined as the number of true positives divided by the number of clinical positive compounds and specificity as the number of true negative compounds divided by the number of negative clinical compounds

The Table is derived from the review by Wallis (2010)

For example, Wallis (2010) evaluated the sensitivity of in vitro hERG, in vitro dog Purkinje fibre and in vivo dog QT assays at 2-, 10- and 30-fold the free ETPC using 11 positive and 8 negative clinical compounds. The results of this study are presented in Table 3. In the Omata et al. (2005) study, it was not possible to compare the specificity and sensitivity of in vitro guinea pig papillary muscle and in vivo dog and monkey QT at 30-fold the free ETPC because the free plasmatic concentrations were not calculated for negative compounds in the in vivo assays.

In this chapter, we undertook to compare the predictive value of in vitro guinea pig action potential to IKr/hERG, dog Purkinje fibre and in vivo dog QT results of the review published by Wallis (2010) at 2-, 10- and 30-fold the free effective therapeutic (clinical) plasma concentration (ETPC). To do that, we based our analysis on the positive in vitro guinea pig supplementary materials of the Hayashi et al. study (2005) and also on the calculated guinea pig EC_{10} values for APD_{90} or $APD_{30–90}$ and reported human unbound ETPC values. We first presented positive in vitro results (at least 10 % increase) for all compounds of the QT PRODACT initiative and the ratio 'in vitro positive concentration/maximal human free ETPC' (Table 4). From these positive concentrations, we could state whether compounds showed positive or negative in vitro results for APD_{90} and $APD_{30–90}$ at 2-, 10- and 30-fold the human free ETPC (Table 5). Positive or negative in vitro results for APD_{90} could not be determined for disopyramide at 10- and 30-fold the free ETPC because the calculated EC_{10} corresponded to fivefold the free ETPC, and because the compound showed a 'bell-shaped' concentration-response relationship, disopyramide was negative at 30 μM, corresponding to 40-fold the free ETPC. It was therefore not possible to determine the free multiple clinical exposure between 5 and 40 where APD_{90} declined down to the 10 % limit and so, turn from positive to negative. For the same reason, the tenfold ETPC result for quinidine was not determined. Regarding the last positive clinical compound thioridazine, we also did not fix a result for the 30-fold safety margin even if result was positive at tenfold (corresponding to the maximal tested concentration of 10 μM in the QT PRODACT initiative). Indeed, the compound is a multichannel blocker showing IC_{50} for INa

and ICaL of 1.83 μM and 1.3 μM, respectively (Mirams et al. 2011), and it also may show a bell-shaped concentration-response relationship and so a negative result at 30-fold the free ETPC. In addition, the maximal tested concentration of negative clinical compounds, ciprofloxacin, flecainide and lidocaine, did not cover the 30-fold safety margin, and it was therefore not possible to determine if results would have been positive or negative at 30-fold the human free ETPC. Finally, from results exposed from Table 5, we determined the specificity and sensitivity of the assay taking into account the safety margins exposed by Wallis in 2010 (Table 6). If the sensitivity of APD_{90} parameter was considered moderate (0.82) when safety margins from clinical exposure were not taken into account in the first published QT PRODACT study (Hayashi et al. 2005), it becomes dramatically insufficient when safety margins of 2- to 30-fold the clinical exposure are considered (max 0.11). Nevertheless, the specificity of the assay is perfect (1.0) at 2-, 10- and 30-fold the clinical exposure. The sensitivity of $APD_{30–90}$ is better than that of APD_{90} for all clinical exposure multiples but nevertheless remains insufficient (0.27, 0.36 and 0.45 at, respectively, 2-, 10- and 30-fold the human free ETPC). On the other hand, specificity for $APD_{30–90}$ declined as safety margin increased from 1.0 at twofold the clinical exposure to 0.75 at 30-fold the clinical exposure. Even if compounds used for the QT PRODACT initiative and those used in the Wallis review (2010) were different, we can conclude from values reported in Tables 3 and 6 that, when compared to IKr, dog Purkinje fibre and dog in vivo QT assays at the recommended 30-fold safety margin (Redfern et al. 2003), the in vitro guinea pig assay exhibits the poorest sensitivity to detect torsadogenic compounds but also the highest specificity.

Table 4 Concentrations inducing at least a 10 % increase in APD_{90} or $APD_{30–90}$ and corresponding calculated EC_{10} (*in italics*) from the in vitro guinea pig papillary muscle action potential assay. The ratio of in vitro positive concentration/maximal human free ETPC is also presented for each parameter (in the right column)

		APD_{90}		$APD_{30–90}$	
	Max unbound clinical ETPC (nmol/L)	Positive in vitro tested concentration and/or *calculated EC10 values (nmol/L)*	Ratio positive and EC10 concentration/unbound human ETPC	Positive in vitro tested concentration *Calculated EC10 values (nmol/L)*	Ratio positive and EC10 concentration/unbound human ETPC
Positive compounds					
Astemizole	0.26	*>10,000*	>35,000	1,000, *2,500*, 10,000	3,500, 9,615, 35,000
Bepridil	33	*>100,000*	>3,030	*3,800*, 10,000, 100,000	115, 303, 3,030
Cisapride	4.9	*200*, 1,000	41,204	*90*, 100, 1,000	18, 20, 204
Disopyramide	742	3,000, *3,900*	*4, 5*	*1,100*, 3,000, 30,000	1.5, 4, 40
E-4031	1.09	*20*, 100	18, 92	*7*, 10, 100	6.4, 9, 92
Haloperidol	3.6	*400*, 1,000, 10,000	111, 278, 2,778	*1,300*, 10,000	361, 2,778
MK-499	0.04	*40*, 100, 1,000	1,000, 2,500, 25,000	*20*, 100, 1,000	500, 2,500, 25,000
Pimozide	0.43	*>10,000*	>23,255	*>10,000*	>23,255
Quinidine	3,237	*6,800*, 10,000	2.1, 3.1	*4,500*, 10,000, 100,000	1.4, 3.1, 31
Terfenadine	0.29	>20,000	>68,966	*>20,000*	>68,966
Thioridazine	979	*7,000*, 10,000	7, 10	*800*, 1,000, 10,000	0.8, 1, 10
Negative compounds					
Amoxicillin	23,841	*>2,700,000*	>113	*>2,700,000*	>113
Aspirin	62,278	*>10,000,000*	>160	*>10,000,000*	>160
Captopril	583	*>100,000*	>171	*>100,000*	>171
Ciprofloxacin	5,281	*>100,000*	>20	*>100,000*	>20
Diphenhydramine	34	*>30,000*	>882	*>30,000*	>882

Flecainide	753	*>10,000*	>13	*5,600*, 10,000	7.4, 13
Lidocaine	6,657[a]	*>100,000*	>15	*>100,000*	>15
Nifedipine	7.7	*>30,000*	>3,896	*>30,000*	>3,896
dl-Propranolol	21.9	*>30,000*	>1,370	*19,200*	877
Verapamil	81	*>100,000*	>1,234	1,000, 10,000, *46,000*, 100,000	12, 123, 568, 1,234

Data has been re-analysed from Hayashi et al. (2005) and Omata et al. (2005)

[a]Note that the maximal free ETPC (not published in the Omata et al. study) was estimated based on a 4 μg/mL plasma concentration and a 39 % unbound plasma fraction (see Collinsworth et al. 1974; Routledge et al. 1980)

Table 5 Positive (+) and negative (−) in vitro guinea pig action potential study results for all QT PRODACT initiative compounds related to safety margins corresponding to 2-, 10- and 30-fold the maximal free effective therapeutic plasma concentration (ETPC)

	APD_{90}			$APD_{30–90}$		
	Clinical exposure multiple (*x*2 free ETPC)	Clinical exposure multiple (x10 free ETPC)	Clinical exposure multiple (x30 free ETPC)	Clinical exposure multiple (*x*2 free ETPC)	Clinical exposure multiple (x10 free ETPC)	Clinical exposure multiple (x30 free ETPC)
Positive compounds						
Astemizole	–	–	–	–	–	–
Bepridil	–	–	–	–	–	–
Cisapride	–	–	–	–	–	+
Disopyramide	–	NA	NA	+	+	+
E-4031	–	–	+	–	+	+
Haloperidol	–	–	–	–	–	–
MK-499	–	–	–	–	–	–
Pimozide	–	–	–	–	–	–
Quinidine	+	NA	–	+	+	+
Terfenadine	–	–	–	–	–	–
Thioridazine	–	+	NA	+	+	+
Negative compounds						
Amoxicillin	–	–	–	–	–	–
Aspirin	–	–	–	–	–	–
Captopril	–	–	–	–	–	–

Ciprofloxacin	–	–	NA	–	–	NA
Diphenhydramine	–	–	–	–	–	–
Flecainide	–	–	NA	–	+	+
Lidocaine	–	–	NA	–	–	NA
Nifedipine	–	–	–	–	–	–
dl-Propranolol	–	–	–	–	–	–
Verapamil	–	–	–	–	–	+

Note that NA indicates that neither a positive nor negative result could be determined

Table 6 Sensitivity and specificity of guinea pig papillary muscle action potential assay to predict the outcome of a clinical QT study. A preclinical result is considered positive for a compound if, at a preclinical concentration exposure (1-, 10- or 30-fold the unbound drug concentrations achieved in the clinic), a minimum 10 % increase in the selected parameter (APD_{90} or $APD_{30–90}$) is observed

	APD_{90}		$APD_{30–90}$	
Clinical exposure multiple	Sensitivity	Specificity	Sensitivity	Specificity
2	0.09	1.0	0.27	1.0
10	0.11	1.0	0.36	0.89
30	0.11	1.0	0.45	0.75

Sensitivity is defined as the number of true positives divided by the number of clinical positive compounds and specificity as the number of true negative compounds divided by the number of negative clinical compounds

3 Conclusion

The QT PRODACT initiative, which investigated the effects of 21 reference compounds on three non-clinical models (IKr, in vitro guinea pig papillary muscle action potential and in vivo dog and monkey QT), gave important information concerning predictive values for these assays to the community of safety pharmacologists. Guinea pig action potential, when tissue is paced at 1 Hz and all tested concentrations are pooled together, was found to be highly specific but moderately sensitive when APD_{90} was the selected predictive parameter, but on the contrary highly sensitive but poorly specific (as it is the case for the IKr assay) when $APD_{30–90}$ was chosen (Hayashi et al. 2005). We have furthermore demonstrated that, considering $APD_{30–90}$, changing the criteria to discriminate positive and negative in vitro results and pacing preparations at faster rates (2.5 Hz) improve the specificity of the assay without altering its sensitivity.

Nevertheless, Omata et al. have shown that comparing EC_{10} values for in vitro guinea pig and IKr IC_{50} or EC_{10} values for in vitro guinea pig and EC_{10} values for dog and monkey in vivo QT, guinea pig was the least sensitive model to detect torsadogenic drugs since higher concentrations of clinical positive drugs were required to induce true positive in vitro results in papillary muscles.

The choice of tested concentrations in the QT PRODACT guinea pig papillary muscle experiments unfortunately did not take into account the concept of safety margin from clinical free ETPC. By refining the sensitivity and specificity of guinea pig action potential from the QT PRODACT initiative data using the integration of clinical multiple exposure, we have also showed that compared to IKr/hERG, dog Purkinje fibres and in vivo dog QT assays, the guinea pig papillary muscle action potential is the most specific but least sensitive model at the well-established recommended 30-fold free ETPC safety margin.

The poor sensitivity of the guinea pig papillary muscle action potential assay should not lead the scientific community to disregard this preclinical model since its specificity should make it interesting. Indeed, it is accepted that there is no existing

preclinical model showing a perfect predictive value for clinical QT and TdP liability. The strategy to adopt is to consider an integrative risk assessment, using a combination of in vitro and in vivo non-clinical models in order to better discriminate between positive and negative clinical compounds (Hanson et al. 2006; Pugsley et al. 2008, 2009; Wallis 2010).

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Haemodynamic Assessment in Safety Pharmacology

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Contents

Abstract

Evaluation of the effects of a drug on arterial blood pressure is important in nonclinical safety pharmacology assessment. Detecting large and obvious changes in blood pressure is an unchallenging task. Detecting small changes is more difficult, and interpretation of findings requires careful risk/benefit evaluation. Detecting subtle and small changes in blood pressure is important in particular with respect to increases, since blood pressure above the normal range is associated with increased risk of stroke and sudden cardiac death.

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Cardiovascular safety pharmacology has been preoccupied with drug-induced changes in the electrocardiogram, and by comparison, there has been little in the way of contemporaneous improvements in the level of complexity and sophistication involved in blood pressure assessment. Thus, it is important to understand the nature of drug-induced changes in blood pressure, appreciate the plethora of agents currently used clinically (and over the counter) that alter blood pressure and understand safety pharmacology study design in order to optimize assessment of a new chemical entity (NCE) or biologic agent in this context.

Keywords
Haemodynamics • Hypertension • Statistics • Study design

1 Drugs Can 'Fail' Because of Effects on Blood Pressure

It is important in safety pharmacology that a spectrum of drug effects be accurately detected, quantified and interpreted (see Pugsley et al. 2008). Since the mid-1990s, the focus in cardiovascular safety has been largely on drug-induced changes on the electrocardiogram (i.e. as they primarily relate to effects on the QT interval). The effects of drugs on systemic arterial blood pressure have not been subjected to the same level of scrutiny, particularly in terms of what to measure, by what means, and how to interpret. This is necessary to provide better assurance that the desired drug efficacy endpoint of the new chemical entity (NCE) or biological therapeutic is distinct from any undesirable or adverse event on blood pressure. The ICHS7A guideline was developed to include the nonclinical evaluation of haemodynamics prior to first in human clinical studies, but the extent of content is small versus the content related to cardiac electrophysiology (Anon 2001).

Arterial blood pressure is a safety pharmacology parameter with a high correlation to adverse cardiovascular clinical outcome in human patients. This is exemplified by findings related to the cholesteryl ester transport protein (CETP) inhibitor (or antidyslipidemic) agent torcetrapib. Torcetrapib was the first CETP inhibitor to be evaluated in phase III clinical development; however, because of 'off-target' effects, torcetrapib elevated blood pressure in patients by a small amount (i.e. ~4 mmHg) and produced an increase in serum aldosterone levels leading to higher cardiovascular events and mortality as described in the ILLUMINATE clinical trial that included 15,057 patients (Psaty and Lumley 2008). Thus, torcetrapib was withdrawn from further clinical development following this observed excess in mortality in the active treatment arm of the study (Howes and Kostner 2007). As safety pharmacologists, we have to ask, why was this liability not detected (or acted upon) in nonclinical studies? Torcetrapib showed positive effects on cardiovascular risk especially in patients with greater increases in HDL-C and apolipoprotein A-1 (apoA-1) levels. However, no nonclinical publications can be found on PubMed prior to conducting of the ILLUMINATE

or other clinical studies conducted concurrently that examined surrogate outcome endpoints including carotid intimal-medial thickness and coronary artery atheroma load (Howes and Kostner 2007). It was not until withdrawal of torcetrapib from clinical development that a series of nonclinical safety studies were conducted (and published) to determine the mechanistic basis for the elevation in blood pressure. Forrest et al. (2008) were the first to attempt to characterize, using nonclinical animal models, the nature of the hypertensive effect of torcetrapib (and compared findings to its analogue, anacetrapib) observed clinically. Both heart rate and blood pressure were measured in telemetered mice and rhesus monkeys as well as anaesthetized rats and dogs. In mice, torcetrapib (10 mg/kg, IV) caused a 15 mmHg elevation in blood pressure, while in rats (at 5 mg/kg, IV), a similar increase was associated with the release of aldosterone and corticosterone. In dogs (10 mg/kg, IV) and rhesus monkeys (500 mg/kg, PO), blood pressure was shown to acutely increase by at least 10 mmHg (Forrest et al. 2008). Anacetrapib was devoid of effects on blood pressure. When Polakowski et al. (2009) dosed torcetrapib both orally (3 and 30 mg/kg) and intravenously (0.01–0.1 mg/kg) to anaesthetized and conscious dogs, both increases in mean arterial blood pressure (of 18–25 mmHg) and heart rates (between 21 and 35 beats/min) resulting from increases in both pulmonary and peripheral vascular resistance were observed. Recently, Johns et al. (2012) provide a very comprehensive overview of the 'on' and 'off' target pharmacology of the CEPT inhibitors citing that the haemodynamic profile of torcetrapib cogeners such as anacetrapib and evacetrapib is distinctly different from torcetrapib and effectively raises HDL-C levels and lowers LDL-C in the absence of off-target activities on blood pressure.

Currently, safety pharmacologists should realize that there are a large number of drugs that are used clinically that are known to cause or exacerbate increases in blood pressure through multiple, usually non-defined, mechanisms of action. These include drugs of abuse (e.g. acute elevation observed with use of 0.625 mg/kg cocaine), sex steroids (e.g. mild, sustained elevation with standard oestrogen contraceptives), non-prescription sympathomimetic drugs (e.g. dose-dependent, sustained increases with phenylephrine and related decongestants), nonsteroidal anti-inflammatory drugs (i.e. mild, sustained increases of ~5 mmHg observed, especially in the elderly), immunosuppressive therapies (e.g. dose-dependent, mild to moderate increases in approximately one third of renal transplant patients treated with cyclosporine A), antidepressants (e.g. monoamine oxidase inhibitors such as tranylcypromine could precipitate a hypertensive crisis) and anti-migraine ergot alkaloids (e.g. dose-dependent increases of >25 mmHg with inhaled ergotamine tartrate (one puff or 0.36 mg)), just to name a few (see reviews by Clyburn and DiPette 1995; Grossman and Messerli 2008; Biaggioni et al. 1990).

Thus, both therapeutic drugs and NCEs (regardless of whether they are small molecules or biological agents) can induce either a transient or persistent increase in systemic arterial blood pressure. Many agents currently in use do not have a defined mechanism of action for effects on blood pressure, and the increase likely results from a multitude of possible physiological changes including altered direct or indirect central and peripheral autonomic tone, altered renal excretion of

electrolytes (i.e. sodium retention) and extracellular volume expansion or direct effects on arteriolar smooth muscle. Similarly, it is also possible that an agent may interfere with the blood pressure-lowering effects of antihypertensive drugs being used by the patient. Thus, as safety pharmacologists, it is necessary to be vigilant with the conduct of the haemodynamic assessment, especially during the development of drugs with potential applicability to use in patient groups recognized to be at higher risk.

The anti-vascular endothelial growth factor (VEGF) signalling agents are an excellent example of a highly efficacious therapeutic class of oncology agents that produce an elevation in blood pressure in patients undergoing therapy for many malignancies. However, oncology is an area not usually encountered by safety pharmacologists during development due to the lack of required stand-alone safety pharmacology studies (by the ICH S9 Nonclinical Evaluation for Anticancer Pharmaceuticals guidance) (Anon 2010). Sunitinib (Sutent, Pfizer), as an example from this class, inhibits cellular signalling of both platelet-derived growth factor (PDGF) and VEGF receptors by targeting multiple receptor tyrosine kinases (RTKs). Thus, by its non-specific action against RTKs, it reduces both tumour angiogenesis and tumour cell proliferation. It is approved for use against renal cell carcinoma (RCC) and imatinib-resistant gastrointestinal stromal tumours (GIST). However, clinicians recognize that hypertension is now a common adverse event with this class of drugs after repeated administration (Di Lorenzo et al. 2009). While overall incidence of hypertension depends upon the agent, responses of between 22 % (for sunitinib) and 32 % [for the anti-VEGF-A receptor monoclonal antibody bevacizumab (Avastin, Genentech/Roche)] have been reported (Grossman and Messerli 2008). The hypertensive response observed is frequently transient and typically resolves upon cessation of therapy (Grossman and Messerli 2008; Ewer et al. 2014). Note that most standard antihypertensive regimens are effective at controlling the observed elevations in blood pressure that result from therapy with this class of oncology agents. This is because RTKs are relevant to both the disease-targeting and to non-target cell biology.

From a nonclinical perspective, the use of animal models can be beneficial to drug safety and subsequent clinical development in the characterization of the haemodynamic safety profile of oncology agents—despite lack of a defined regulatory requirement. Use of the conscious, unrestrained rat telemetry model has been reported to be a predictive model with which to identify the hypertensive response that may be manifest by anti-VEGF signalling inhibitors after repeat dose administration (Curwen et al. 2008; Isobe et al. 2014). Use of this standard safety pharmacology model for haemodynamic assessment of cediranib, sunitinib and sorafenib suggests that the response profile or trend to development of hypertension was similar to that observed in clinical studies which illustrates the potential value of preclinical screening models in drug safety testing. One should take account of the fact that because of the high mortality rates observed with cancer, development of anticancer drugs requires minimal, if any, nonclinical safety pharmacology data for regulatory authorities to determine whether marketing authorization should be granted; rather this is based upon clinical trial outcomes.

2 Clinical Trends in Cardiovascular Disease: A Focus on Blood Pressure

According to statistics from the American Heart Association (AHA), it can be estimated that more than one third of American adults have at least one variant form of cardiovascular disease (CVD). CVD is an intricate, highly integrated class of dysfunction that is comprised primarily of a multitude of disease pathologies defined as hypertension, coronary heart disease, heart failure and stroke. Our concern in this chapter involves characterizing drug safety for NCEs and biologicals that may affect blood pressure; thus, it is important that the safety pharmacologist understands the prevalence of hypertension in man. Note that currently, no means of gauging risk observed from elevated blood pressures in nonclinical studies can be translated to the clinic despite the fact that from examples of drugs currently known (e.g. anti-VEGF signalling inhibitors), elevations in blood pressure in animals can have similar effects in humans. It is the lack of apparent translatability of the magnitude of the altered blood pressure response that remains the unknown in evaluating the risk of meaningful adverse clinical effects.

In the general population, essential hypertension is recognized as an altered relationship between cardiac output and vascular resistance and is clinically defined as a systolic blood pressure of >140 mmHg and a diastolic blood pressure of >80 mmHg. In general, 34 % of Americans suffer from some variant form of hypertension as outlined in the Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure (JNC 7) (Chobanian et al. 2003). The prevalence of hypertension appears comparable between genders; however, it varies between Caucasians and African Americans. African Americans tend to have the highest reported rates of hypertension (~45 %) in the world (readers are referred to Pugsley et al. 2010 for details). Additionally, studies have been conducted that examine age-specific relevance of blood pressure to cause-specific mortality. The deduction of such cause and effect is based on conduct of a meta-analysis of prospective observational studies of blood pressure and mortality clinical trial data that has identified a direct correlation between arterial blood pressure and mortality. The study suggests that the proportional risk in vascular death does not change to values of at least 115/75 mmHg for systolic and diastolic blood pressures (Lewington et al. 2002). In further characterizing the clinical landscape, the prevalence of hypertension increases to more than 66 % at 60 years and older (Nwankwo et al. 2013). The high prevalence of hypertension in both the adult and elderly populations adds to the risk of the potential for increased incidence of cardiovascular adverse events with drugs that increase arterial blood pressure. Because increases in blood pressure can constitute a disease process with increased risk of death, any drug that increases blood pressure should be appraised in this context when safety assessment is undertaken.

Thus, in general, most hypertensive patients have essential hypertension or other better known forms of secondary hypertension that could include renovascular hypertension (due to renal artery stenosis), or chronic renal insufficiency (due to renal parenchymal disease), adrenal or steroid abnormalities (due to

hyperaldosteronism) or pheochromocytoma. Because most clinicians and patients are less aware of other secondary forms of hypertension including an elevation in blood pressure due to drugs, it is critical in the safety pharmacology assessment that an understanding of the effects of the NCE or biological be determined and included in the integrated risk assessment. Thus, it is important to understand the structural components of the vascular system involved in the maintenance of blood pressure. This will be briefly reviewed.

3 Basic Structural Features of the Vascular System

The vascular system of the human body is comprised of a large number of vessels that play an integral role in the movement of blood through the circulatory system. It is only through the extensive network of arteries, capillaries and veins that cellular function, absorption of essential nutrients and removal of cellular and metabolic wastes can be maintained. Thus, despite such diversity of physiological function, it is necessary that vessels and related structural elements involved exhibit some homology of cellular structure and composition yet retain distinctive cellular properties that ensure proper physiological function is maintained (Pugsley and Tabrizchi 2000).

The development of research methodologies for use in determining drug effects on cardiovascular haemodynamics requires an understanding of some of the basic cellular physiological properties of the system. The vascular system, despite the species and complex regional and organ differences, shares a common histological organization.

Most blood vessels consist of three histologically distinct regions in which each region contains variable amounts of smooth muscle cells and elastin. The variability in cellular constituent is based upon physiological function that the vessel serves to an organ or tissue. Each region in a blood vessel is termed a '*tunic*' and anatomically constitutes the *tunica intima*, *tunica media* and *tunica adventitia*. The tunica intima consists on the luminal side of the vessel and consists of a single layer of endothelial cells on a basement membrane. Below this membrane exists a sub-endothelial fibro-elastic layer of connective tissue and organized internal elastic lamina that provide the endothelial cells with stability and flexibility. Perivascular cells (or pericytes) are present in apposition to endothelial cells which provide a balanced microenvironment. Endothelial cells perform a critical role in all aspects of tissue homeostasis including regulation of vascular tone, thrombosis and haemostasis, inflammation and immunological processes (Pugsley and Tabrizchi 2000).

The *tunica media* contains predominantly smooth muscle cells and elastin fibres that are much more highly organized in larger arteries due to the role required in the movement of large volumes of blood. Additional structural support is provided by an external elastic lamina. The remaining and outermost layer of the blood vessel is the *tunic adventitia* that is composed almost entirely of fibro-elastic connective tissue. Lymphatic and nerve plexi are found in the *adventitia* along with the *vasa*

vasorum. The *vasa vasorum* is a network of small thin-walled blood vessels that essentially supply the walls of large blood vessels (veins and arteries) with adequate nutrients and oxygen.

3.1 The Arterial and Venous Components of the Circulatory System

The function of the left ventricle is to eject oxygenated blood returning from the lungs into the arterial tree. Arteries are histologically classified into two types: elastic and muscular (Borysenko and Beringer 1984). Elastic (or conducting) arteries are present close to the heart or organs associated with the movement of large volumes of blood. These arteries are composed of many layers of perforated elastic membrane and are therefore particularly adapted to accommodate large changes in blood volume such as those typically associated with ejection from the left ventricle of a beating heart. The highly elastic nature of the walls of these blood vessels effectively 'damp' large oscillations in blood flow and provide for a more homogeneous movement of blood away from the heart. The function of muscular (or distributing) arteries is to ensure blood is rapidly and completely distributed to all organs and tissues. While the walls are predominantly composed of smooth muscle cells, these arteries do contain discontinuous elastic fibres.

The continuous bifurcation of arteries within the cardiovascular system leads to blood flow within the smallest blood vessels of the arterial tree, the capillaries. The function of these important arterioles is to limit blood flow from the larger arteries in order to prevent damage to the fragile capillaries that connect the arterial vascular tree to the venous system. The capillaries also regulate, through a complex interaction with autacoids, hormones and neurotransmitters, the microcirculation by altering arterial smooth muscle wall tone (Pugsley and Tabrizchi 2000).

The arterial system transitions to the venous system through the capillary network. The capillary is histologically distinct in that it lacks smooth muscle and instead consists of a single layer of endothelial cells, a basement membrane, and is enveloped in a population of pericytes (Hirschi and D'Amore 1996). It is across the large surface area associated with the capillary bed that nutrients, solutes and water are exchanged via simple diffusion according to solute concentration gradients and hydrostatic and osmotic pressures between blood and the surrounding tissues. Several types of capillaries can be histologically distinguished. These include the continuous (the most ubiquitous in the body associated with most organs) capillary, the fenestrated (present in endocrine organs and in the renal glomeruli) capillary and the discontinuous (present in bone marrow and spleen) capillary.

Venous return of blood to the heart starts with its movement through the post-capillary venules, which coalesce to form larger veins. Histologically, the transition from capillary to vein is marked by the measured appearance of smooth muscle cells within the *tunica media* of the vessel wall and collagen and elastic fibres within the *tunica adventitia*.

Table 1 A list of applicable methods for use in the study of many aspects of haemodynamics and blood vessel function in nonclinical safety and efficacy pharmacology studies

In vivo methods	In vitro methods
Flow probes • Doppler • Electromagnetic • Ultrasonic	Isolated tissue preparations • Arterial (rings, spiral strips, perfused) • Veins (intact/denuded endothelium)
Microsonometry	Isolated cell methods • Freshly dissociated smooth muscle cells • Cultured vascular endothelial cells
Intravascular ultrasound	
Vascular imaging	
Angiography	Electrophysiology
Indicator dye dilution	Biochemical methods
Radiotelemetry	Immunohistochemical (IHC) staining
Tail cuff plethysmography	
High-definition oscillometry (HDO)	
Intra-arterial catheters	

Note that the methods outlined in this table are based on those used by the authors in the past to investigate drug effects on the haemodynamic/vascular system in an investigative manner to resolve an off-target-related drug effect. Use of these methods is not outlined or discussed in the ICHS7A guidance document nor should their application in a drug safety investigation be considered a regulatory requirement

These basic histological properties of the blood vessel are important in understanding the complexity of blood flow and how it is different between arteries and veins. It is by no means a complete overview, and readers are referred to standard physiology textbooks that describe physical properties of flow such as fluid dynamics. Such differences between an artery and vein are important if the safety pharmacologist is to correctly measure flow in these systems. Table 1 provides a list of both in vivo and in vitro methods that may be utilized to investigate effects of a novel drug on blood pressure. Note that these methods remain investigational and are not sanctioned in the ICHS7A guidance document rather they reflect the various types of methods that can be used to assess drug-induced changes in blood pressure. Their usage and relevance to the adverse event observed in drug development remain a decision of the safety pharmacologist and nonclinical development and/or discovery sciences team.

4 Safety Pharmacology Study Design and Statistical Consideration

It is standard practice in safety pharmacology to conduct a power analysis (i.e. establishes the sensitivity of the statistical test or the ability of a test to detect an effect, if the effect exists) by using data derived from each nonclinical model in order to assess the minimum detectable difference (MDD) for the parameter to be investigated. With regard to blood pressure changes, an effect size of 14–16 % (Ewart et al. 2013) was calculated as the MDD for haemodynamic measurements

from conscious telemetered dogs (note that $n = 4$ was used with 80 % power analysis). Using canine normal mean arterial blood pressure values, this translates to a ~17 mmHg difference—which is far greater than the observed 4 mmHg change in arterial blood pressure reported to be responsible for adverse cardiovascular effects observed in clinical trials with torcetrapib. However, when similar canine telemetry data was analysed using super-intervals (i.e. when analysed over larger summary periods such as hours vs. minutes), the MDDs were decreased to 5 mmHg and 10 beats per min in conscious dogs (note that an $n = 4$ was used with a similar 80 % power analysis) (Sivarajah et al. 2010). However, super-intervals also have limitations, mostly with any NCE that presents transient effects that then prevent data averaging over longer summary periods. The major risk with the application of super-intervals to telemetry data is the potential to overlook a pharmacodynamic effect due to averaging with unaltered data. The other challenge with super-interval analysis is to predict the timing of drug effect as super-intervals are selected to distinguish periods with haemodynamic changes from those without drug effects. As the pharmacodynamic profile of a drug is typically unknown when the safety pharmacology study is initiated, selecting the most appropriate super-intervals can be difficult. Pharmacodynamic changes often mirror pharmacokinetic exposures, and the latter usually serves as a basis for selection of the super-interval. As illustrated by the effect of choosing the super-interval duration on MDD, refinement of the statistical analysis plan represents an approach with which to increase the sensitivity of nonclinical safety pharmacology models. Other possible strategies to enhance the statistical sensitivity of haemodynamic models include the application of statistical analysis with a 24 h data average as a covariate or using a time-matched control (see Sethuraman and Sun 2009). Multiple methods to improve the statistical analysis are available to safety pharmacologists and can be included when designing the experiment in consultation with statisticians.

Beyond statistical analysis, individual study values need to be evaluated for possible drug effects that may only affect a subgroup of treated animals. In order to appreciate individual animal study values that may be changed by an NCE or biologic drug during development, it is critical that the safety pharmacologist understand the magnitude of the control or baseline (i.e. nondrug treated) values that are common for the study parameters being investigated in different animal species. A review of the data in Tables 2 (rhesus monkeys), 3 (cynomolgus monkeys), 4 (Beagle dogs) and 5 (Sprague-Dawley rat) provides examples of values that can be acquired for standard EKG, blood pressure and cardiac contractility parameters in conscious, telemetered, acclimated nonclinical animal species that may be used in safety pharmacology blood pressure (or other cardiovascular) studies. Biological variability is expressed in most safety pharmacology studies, and the presence and magnitude of the response are often different between individuals (as in the clinic with patients). However, one should be reminded that regulatory authorities expect the potential for a clinically significant change in a haemodynamic parameter to be discussed within the study report, even if statistical significance is not achieved.

Table 2 A list of typical control EKG and blood pressure values obtained from telemetered rhesus monkeys

	Daytime		Night-time	
Rhesus monkey telemetry data	Mean	SEM	Mean	SEM
PR (ms)	97.7	0.9	97.1	0.8
QRS (ms)	32.5	0.2	33.0	0.1
QT (ms)	202.5	2.3	264.9	3.6
HR (BPM)	158.5	3.8	105.5	1.2
QTcB (ms)	327.1	1.2	348.5	2.8
Body temperature (°C)	38.2	0.1	36.4	0.1
Systolic arterial blood pressure (mmHg)	127.7	2.1	102.7	0.4
Mean arterial blood pressure (mmHg)	104.3	2.0	79.0	0.7
Diastolic arterial blood pressure (mmHg)	81.0	1.7	58.2	0.8

Data are from $N = 10$ animals

Table 3 A list of typical control EKG and blood pressure values obtained from telemetered cynomolgus monkeys

	Daytime		Night-time	
Cynomolgus monkey telemetry data	Mean	SEM	Mean	SEM
PR (ms)	75.9	0.9	78.2	0.8
QRS (ms)	32.5	0.3	32.5	0.2
QT (ms)	209.3	4.0	226.8	3.9
HR (BPM)	163.1	3.8	115.8	3.3
QTcB (ms)	339.7	3.4	345.7	3.0
Body temperature (°C)	37.6	0.1	36.9	0.1
Systolic arterial blood pressure (mmHg)	116.8	1.5	115.3	1.2
Mean arterial blood pressure (mmHg)	97.7	1.2	96.4	1.1
Diastolic arterial blood pressure (mmHg)	80.5	1.1	79.6	1.0

Data are from $N = 14$ animals

The use of an anaesthetized animal model may be considered to increase the sensitivity to detect drug-induced haemodynamic changes. Under anaesthesia, cardiovascular parameters tend to be more stable (Authier et al. 2008) which results in lower MDDs. However, the use of anaesthesia also results in a depression of compensatory mechanisms and may, depending upon the nature of the anaesthetic, actually increase the magnitude of a haemodynamic response compared to the same conscious animal model. There are disadvantages to the use of anaesthetized models for haemodynamic assessments in safety pharmacology. First, the response obtained under anaesthesia may differ from the target clinical population typically composed of conscious individuals (see Fig. 1a, b). In Fig. 1a, the effects of remifentanil, a potent ultrashort-acting synthetic opioid analgesic drug, are strikingly different in conscious animals where there is a minimal blood pressure

Table 4 A list of typical control EKG, blood pressure and cardiac contractility values obtained from telemetered Beagle dogs

Beagle dog telemetry data	Daytime		Night-time	
$n = 16$	Mean	SEM	Mean	SEM
PR (ms)	109.3	1.0	110.0	0.6
QRS (ms)	36.9	0.1	36.4	0.1
QT (ms)	214.0	2.4	220.2	1.0
HR (BPM)	101.4	3.7	85.0	2.1
QTcV (ms)	244.7	1.3	242.6	1.2
QTcF (ms)	249.9	1.6	244.7	1.3
Body temperature (°C)	37.4	0.1	37.4	0.0
Systolic arterial blood pressure (mmHg)	152.9	3.3	147.0	1.4
Mean arterial blood pressure (mmHg)	106.8	2.7	100.3	1.2
Diastolic arterial blood pressure (mmHg)	82.3	2.2	77.5	1.1
Left ventricular diastolic pressure (mmHg)	20.9	0.7	21.2	0.8
Left ventricular systolic pressure (mmHg)	168.2	3.1	150.7	2.2
Contractility (1/s)	77.5	3.2	72.8	1.3
Vmax (1/sex)	1,478.3	41.3	1,245.7	30.9
Max dP/dt (mmHg/s)	3,438.5	124.9	2,832.8	68.6
Min dP/dt (mmHg/s)	−4,036.0	111.8	−3,543.2	36.1
Tau (ms)	57.0	3.7	57.1	0.9

Data are from $N = 16$ animals

Table 5 A list of typical control EKG, blood pressure and cardiac contractility values obtained from telemetered Sprague-Dawley rats

Sprague-Dawley Rat telemetry data	Daytime		Night-time	
$n = 16$	Mean	SEM	Mean	SEM
HR (BPM)	338.4	3.3	381.7	3.9
Body temperature (°C)	37.1	0.1	37.8	0.1
Systolic arterial blood pressure (mmHg)	125.6	1.9	128.1	1.9
Mean arterial blood pressure (mmHg)	106.9	1.8	110.1	1.8
Diastolic arterial blood pressure (mmHg)	91.0	1.7	95.3	1.8
Contractility (1/s)	146.0	0.5	167.4	2.3
Vmax (1/sex)	2,740.5	598.5	2,647.5	910.4
Max dP/dt (mmHg/s)	7,693.0	382.3	8,934.3	577.0
Min dP/dt (mmHg/s)	−6,343.7	404.9	−7,161.1	516.4
Tau (ms)	17.0	3.7	57.1	0.9

Data are from $N = 16$ animals

response compared to the marked hypotensive response in anaesthetized animals. In contrast, infusion of dopamine, a catecholamine and phenethylamine family neurotransmitter, in conscious animals produced a minimal (<10 %) increase in blood

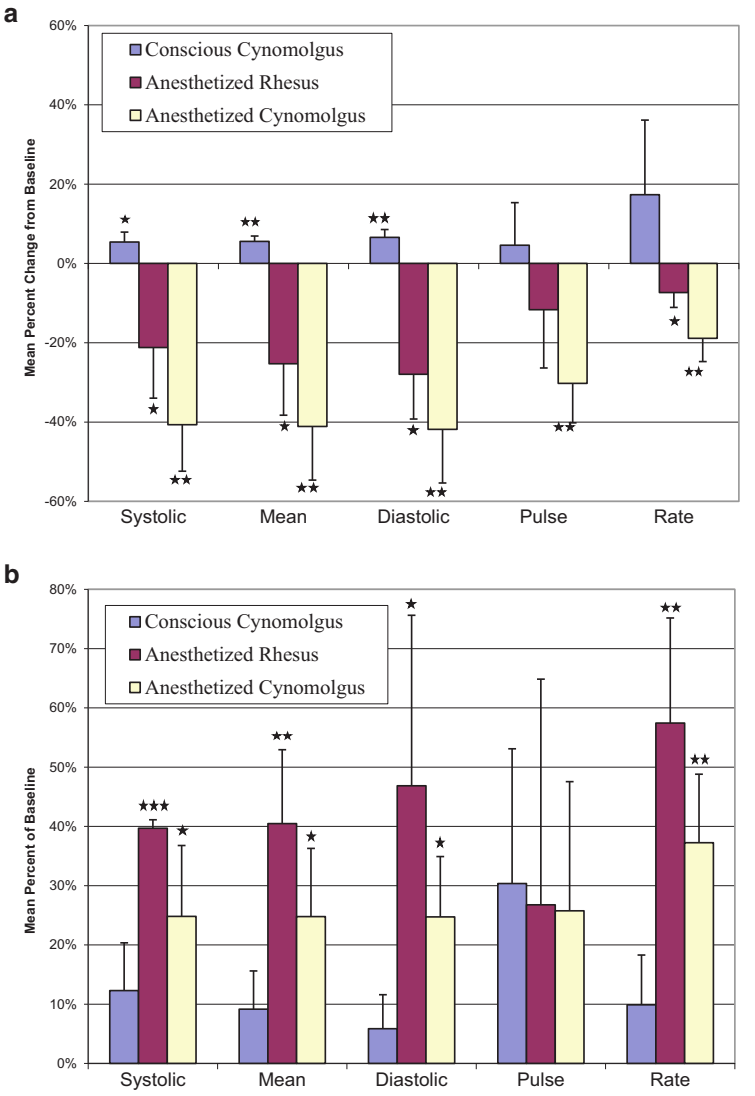

Fig. 1 (**a**) Haemodynamic effects of the opioid analgesic remifentanil (4.0 μg/kg, IV) administered to either conscious telemetered cynomolgus monkeys or anaesthetized (1.5–2 % isoflurane) cynomolgus and rhesus monkeys. Note that $*p < 0.05$ and $**p < 0.01$ denote statistical significance versus baseline. (**b**) Haemodynamic effects of dopamine (0.05 mg/kg/min, IV) administered to either conscious telemetered cynomolgus monkeys or anaesthetized (1.5–2 % isoflurane) cynomolgus and rhesus monkeys. Note that $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ denote statistical significance versus baseline

pressure, however, in anaesthetized animals produced marked increases (>25 %). Thus, these potential effects are understood and emphasized in the safety pharmacology guideline ICHS7A which recommends the use of conscious models (Anon 2001). Anaesthesia attenuates central nervous system responsiveness which may result in pharmacodynamic effects that differ between conscious and anaesthetized animals (Authier et al. 2008). As another consideration, parenteral dosing is normally required in anaesthetized models due to inhibition of peristaltic activity in the gastrointestinal system by most anaesthetic agents (Ng and Smith 2002; Torjman et al. 2005). Reduced peristaltic activity usually prevents adequate absorption with oral dosing. In addition to possible changes in body temperature, hormonal balance, cardiac output or vascular tone, the use of anaesthesia may also affect metabolism and elimination of a test drug, potentially altering the pharmacodynamic profile obtained in such a model when compared to conscious animals. Continuous monitoring of body temperature is recommended with all models given the known effects of changes in core body temperature on haemodynamic (and EKG) parameters. When using anaesthetized models, maintaining body temperature within ± 0.5 °C of the normal temperature with adjustable heating devices is ideal to maximize reproducibility. Anaesthesia with an inhalant gas (e.g. isoflurane or sevoflurane) will cause water loss through respiratory airways. Replacement fluids warmed to normal body temperature and delivered intravenously at a rate of 10 mL/kg/h are often valuable to maintain circulating blood volume. Even with the rigorous control of normal body temperature and fluid replacement, most agents used for anaesthesia are also associated with a progressive decrease in haemodynamic values which prompts for comparisons with data obtained at time-matched intervals after vehicle treatment. Overall, anaesthetized models are very sensitive in their ability to detect very subtle haemodynamic changes with parenteral dosing but may not identify (vaso)pressor effects mediated at least partially by the central nervous system.

It is common practice to use anaesthetized models, often rodents, at an early stage of drug safety testing (i.e. cardiovascular frontloading). The small size of rodents enables evaluation of potential cardiovascular and/or cardiac liabilities with a limited quantity of compound. With the high sensitivity to haemodynamic effects in anaesthetized animals, studies with relatively small group size (e.g. n usually 3–4) can be cost-effective and rapid to complete. It is difficult to apply proper statistical methodologies to group sizes below $n = 5$ which means that if the decision is to halt development of a drug, this may be erroneous. If the decision is to halt development of a class of drugs, it requires similar (albeit nonstatistically evaluable) effects in order to be confident that the class is not being rejected erroneously. If the decision is to proceed with the NCE, then this is hazardous, and increasing group sizes to be more confident of the data is mandatory.

Most safety pharmacology studies with haemodynamic assessments may include a control treatment group tested under the same conditions of the drug being considered for development. The presence of a control group is particularly important for statistical analysis. However, it remains that some designs may not require a dedicated control group. Early safety screening studies in anaesthetized rodents or

maximum tolerated dose (MTD) studies that aim to determine, if possible, the threshold at which adverse haemodynamic changes occur may be successfully conducted without a dedicated control group. It is important to remember that when it becomes clear that an adverse drug reaction liability is detected, the owner of the drug is likely to opt to end research on the drug. This decision does not require publication of findings and likewise does not require fully powered studies and reliable statistics. The key issue here is that the owner of the drug must decide what is sufficient to justify ending a drug or class development. In contrast, when the owner decides to continue development, it becomes more important to have fully powered persuasive data sets because decision making (marketing authorization or approval of whatever type) will be made by others. All other considerations are technical.

Ultimately, study design integrates a broad range of considerations including the target clinical population (and tolerance to adverse effects), pharmacokinetics, the development stage at which the study is conducted or prior data obtained with the drug candidate.

5 Logistical and Technical Considerations in the Conduct of Haemodynamic Assessments

The quality of haemodynamic data is directly proportional to the precautions taken during acquisition of the data. A systematic review of refinement strategies in the conduct of cardiovascular safety pharmacology studies, although simplistic in appearance, is a pre-requisite to proper data interpretation and later to decision making in drug development. The following discussion will cover a number of logistical considerations that may impact haemodynamic data quality and review possible tactics to aid refinement.

Circadian cycles are observed in most cardiovascular animal models (Soloviev et al. 2006). To achieve the lowest minimum detectable difference for haemodynamic parameters, administration of the test drug or vehicle/control is required precisely at the same time of the day to enable time-matched comparisons between treatments. As illustrated in Fig. 2a, b, dosing at different times during the day may lead to erroneous cardiovascular data interpretation (Fig. 2a), while the time-matched data comparison (Fig. 2b) ensures that changes caused by simple circadian cycle overlap between sessions can be accounted for properly in the study. Particular attention should be paid to studies that overlap between periods with changes in daily saving time (i.e. summer and winter day times) as a 1 h shift in light cycle creates an adaptation period that affects hourly means for various parameters including haemodynamic assessment. Furthermore, feeding and cage cleaning also introduce changes in haemodynamic parameters. Feeding or any other procedure that requires the presence of the technical staff in the animal room should be minimized and when needed should be scheduled precisely at the same time each day. Specifically, husbandry typically has an impact on cardiovascular parameters and should be performed preferentially outside the monitoring period in order to

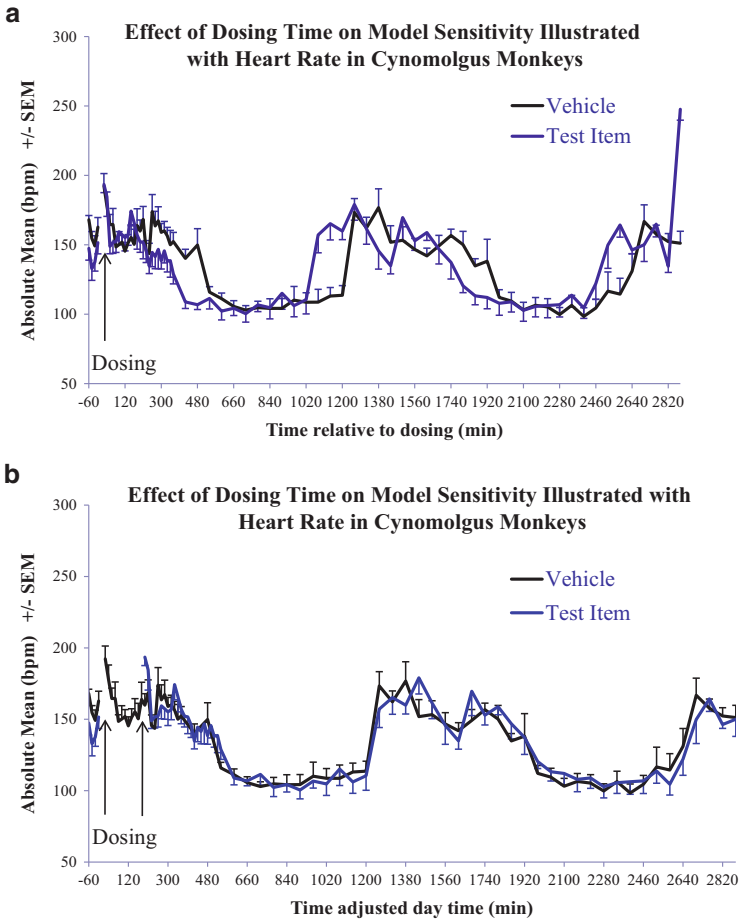

Fig. 2 (**a**) Heart rate data from cynomolgus monkeys presented relative to dosing time. Dosing with vehicle was completed 120 min prior to the test item administration. A comparison of data captured at different times of the day during data analysis may lead to an incorrect interpretation of drug effects. (**b**) Heart rate data from cynomolgus monkeys adjusted for the time of day. Dosing with vehicle was conducted 120 min prior to the test item administration but only data collected at the same time of day was used for comparison. Dosing time should be precisely maintained between groups to enable accurate comparison of drug effects

minimize artefacts due to stress. If the duration of the cardiovascular data recording usually exceeds 36 h, cage cleaning should be planned precisely at the same time of the day after all treatments to allow time-matched comparisons.

Progressive acclimation to experimental procedures is usually observed in animal models, and haemodynamic parameters are most sensitive to such environmental interferences. When selecting the optimal study design, this progressive change in baseline conditions needs to be considered. In this context, a dose

escalation paradigm in the same animals normally presents lower sensitivity than a cross-over or a Latin square design.

Behavioural interactions from drug-treated animals on parameters recorded in other individuals from the same study but receiving different treatments represent an underestimated source of interactions and variability. Treatments causing substantial excitatory effects can affect haemodynamic parameters in vehicle-treated animals housed in the same room. Similarly, drugs disrupting sleep patterns can impact all animals in a room through perturbation in the sleep cycle of all individuals in the room if one is overly active or vocalizing.

For all intravenous injectable drug products, maximal exposure is observed immediately at the time of administration. Most parenteral dosing routes (e.g. subcutaneous and intramuscular) are associated with an early maximal concentration (C_{max}). As previously discussed, the presence of study personnel in the animal room creates important artefacts that impinge upon baseline haemodynamic values. Dosing systems that enable remote dosing (e.g. use of ambulatory pumps or jackets with swivel and tethers for bolus dose administration) can be used to significantly improve the quality of haemodynamic data avoiding the presence of staff in the animal room, especially during the immediate post-dose period. When using remote dosing systems with continuous intravenous infusion, maintaining an infusion with vehicle at the same rate as the drug treatment may be appropriate for short-acting drugs to avoid haemodynamic effects attributed to rapid changes in the infusion rate. Of course, dosing systems where the staff can access the remote dosing infusion pumps and lines without visual and auditory contact with the animals are preferable.

Volumes, dose rate (bolus or infusion) and physicochemical characteristics of the vehicle or test article solutions are critical factors that may impact haemodynamic results. Using the lowest dose volume possible reduces the risk of haemodynamic changes caused by a change in circulating blood volume at the time of injection. Dosing volume maintained at less than 2 mL/kg delivered as a short infusion (e.g. 5 min or longer) is typically well tolerated and will not introduce major fluctuations in arterial blood pressures. Osmolality is ideally maintained as close as possible to the physiological range for plasma (i.e. 275–295 mOsm/kg) to avoid hyper- or hypo-osmotic effects. An osmolality above 600 mOsm/kg is considered high risk with regard to precipitation of negative effects on erythrocytes due to hyper-osmotic effects. Dosing formulations with low osmolality (i.e. 180–275 mOsm/kg) are typically well tolerated when given acutely, but chronic infusion of hypo-osmotic formulations may be associated with negative effects on clinical chemistry parameters and subsequently on cardiovascular function. Hypo-osmotic formulations may also cause erythrolysis due to diffusion of free water from the plasma into erythrocyte to re-establish neutral osmolality causing an overt increase in intracorpuscular volume possibly leading to destruction of the red blood cells. Hyper-osmotic formulations [e.g. as may be found with a high percent of dimethyl sulfoxide (DMSO)] may also cause erythrolysis even with low doses. Whenever possible, the pH of dosing formulations should be adjusted to the physiological range (i.e. 7.35–7.45). Acidic formulations are better tolerated than basic solutions

when delivered intravenously. Mammals have a range of natural mechanisms that are used to eliminate acidic by-products from oxidative or anaerobic metabolism. The buffer capacity of plasma proteins and circulating bicarbonates represents two important pathways to rapidly neutralize acidic dosing formulations. These natural adaptations may explain the higher tolerance for acidic dosing formulations compared to basic solutions. When using low-dose volumes (e.g. <1 mL/kg), dosing formulation at pH 4.5–7.35 is typically well tolerated. The buffer capacity of the solution is another factor that may impact tolerability. Formulations with low pH (e.g. 3) but with low buffer capacity may be well tolerated. Basic formulations with pH from 7.45 to 8.5 are typically well tolerated, but higher pH (i.e. 9 to 11) will typically cause signs of alkalosis with adverse consequences. The ideal dosing formulation for haemodynamic assessments of a drug candidate is often a compromise between efforts to maintain the dose volume as low as possible while keeping osmolality and pH close to the physiological range. It is generally more important to maintain osmolality and pH within acceptable ranges than to reduce the formulation dose volume. Larger dose volumes (e.g. 2–4 mL/kg) can be delivered with minimal impact on haemodynamic parameters using a longer infusion (e.g. over 30 min), but adverse effects due to extreme pH or osmolality cannot be mitigated by changing the dosing regimen. When using organic solvents, high-dose volumes or pH/osmolality outside of the standard physiological range, a staggered dosing regimen with the control group delivered first can be planned to ensure suitability of the formulation and dosing regimen selected.

Quality of the haemodynamic data is also affected by technical factors. Major factors to consider include the possibility of hydrostatic pressure effects (i.e. blood inside the vasculature or fluid-filled catheter systems that are utilized in recording methods), long-term stability of the pressure sensor (subject to possible drift with time), frequency response of the sensor and associated modern solid-state electronics (see Sarazan 2014 for a complete review of technological requirements and potential errors that can be encountered when measuring cardiovascular pressure in safety pharmacology studies).

6 Interpretation of Haemodynamic Data in Safety Pharmacology Studies

While statistical analysis is often the main support to haemodynamic data interpretation, careful review of individual animal data may allow identification of drug effects that may not reach statistical significance. While a majority of clinical trials are designed with appropriate statistical power, most safety pharmacology studies include a limited number of animals (e.g. usually $n = 4$) that may not be sufficient to achieve statistical significance even in the presence of moderate or even marked drug-induced effects since there may be an associated high degree of variability within the small group size (see discussion above). It remains that drug effects that do not reach statistical significance, potentially due to interindividual biological variability, may still be identified at data review when present in a few individuals

in the group. Caution is usually warranted during drug safety data interpretation when moderate to severe haemodynamic effects are observed in a limited number of animals, and this is discussed as a potential drug-mediated effect. A drug effect only noted in a subgroup of animals may theoretically represent a portion of the intended target clinical population and may subsequently be confirmed to be clinically significant in humans.

Comparison of haemodynamic data from time-matched periods for each animal is a common approach during qualitative evaluation of potential drug effects and requires sufficient pre-dose or vehicle/control data to support interpretation. While the same time of day remains the most appropriate control period for interpretation, variations when comparing different days at the same time are still noted in all species with non-rodents presenting higher variation due to diverse behavioural activities. Rodents housed in a laboratory environment exhibit relatively low levels of physical activity which potentially translates into lower levels of haemodynamic change that can be observed.

Haemodynamic parameters are influenced by numerous physiological factors. Positive chronotropic effects (i.e. increases in heart rate) are expected to cause an associated increase in arterial blood pressure primarily resulting from an increase in cardiac output. Figure 3 depicts the correlation between heart rate and $+dP/dt_{max}$, the maximal rate of rise of left ventricular pressure (LVP) that is determined by myocardial contractility and cardiac loading conditions on the ventricle (see Hamlin and del Rio 2012) when dopamine is administered intravenously to conscious telemetered rats.

Similarly, neurological stimulation will typically be associated with an increase in arterial blood pressure due to an increase in physical activity and associated

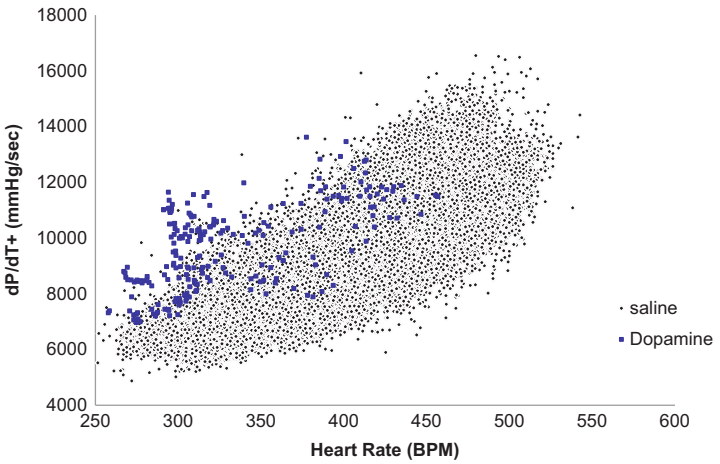

Fig. 3 The correlation between heart rate and $+dP/dt_{max}$ (the maximal rate of rise of left ventricular pressure) when a positive cardiac inotropic agent such as dopamine (0.05 mg/kg, IV) is administered to conscious telemetered rats

changes in heart rate and vascular tone. Dehydration (e.g. due perhaps to diuretic effects) and non-specific toxicological effects observed in drug safety studies are often noted to cause negative haemodynamic effects.

When drug-induced vasodilation is present, a compensatory increase in heart rate is expected subsequent to a decrease in arterial blood pressure and may help confirm the presence of a drug-induced pharmacological effect. A decrease in arterial blood pressure combined with a decrease in heart rate is likely a consequence of a cardiodepressive effect with negative chronotropy or decreased myocardial contractility.

7 Conclusion

In conclusion, there is a body of knowledge and expertise to inform intelligent safety pharmacology research on blood pressure adversely affecting liability. It important that investigators make use of this knowledge when designing and interpreting studies that investigate drug-induced changes in blood pressure. An additional understanding of the spectrum of methods available for use in many different animal species to aid in the investigation of 'off'-target drug-mediated changes in blood pressure is also critical to the safety pharmacologist since there exists a multitude of mechanisms that ultimately control blood pressure.

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High Definition Oscillometry: Non-invasive Blood Pressure Measurement and Pulse Wave Analysis

Beate Egner

Contents

Abstract

Non-invasive monitoring of blood pressure has become increasingly important in research. High-Definition Oscillometry (HDO) delivers not only accurate, reproducible and thus reliable blood pressure but also visualises the pulse waves on screen. This allows for on-screen feedback in real time on data validity but even more on additional parameters like systemic vascular resistance (SVR), stroke volume (SV), stroke volume variances (SVV), rhythm and dysrhythmia. Since complex information on drug effects are delivered within a short period of time, almost stress-free and visible in real time, it makes HDO a valuable technology in safety pharmacology and toxicology within a variety of fields like but not limited to cardiovascular, renal or metabolic research.

Keywords

Cardiovascular • Cats • Common marmosets • Cynomolgus • Diabetes • Dogs • HDO • High-definition oscillometry • Metabolic • NIBP • Pulse transit time

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(PTT) • Pulse wave analysis (PWA) • Research • Safety pharmacology • Toxicology

Approximately 40 % of the human population is affected with hypertension with increasing tendency. In the elderly, hypotension can also become a severe problem. Since diabetes and metabolic syndrome are more and more in the focus, early detection of changes in the vascular system becomes increasingly important. In kidney and heart disease, emphasis is made to find evidence of altered systemic vascular resistance (SVR) early, non-invasively and stress-free.

The effects drugs have on blood pressure (BP), stroke volume (SV), stroke volume variances (SVV) and SVR need to be taken into account as early as possible in drug development but especially in safety pharmacology and toxicology studies. Non-invasive techniques to date have, however, had numerous limitations which restrict more frequent monitoring of these vitally important parameters. High-Definition Oscillometry (HDO) was developed in 2006 and used in research since 2008. It proved to be highly accurate and reliable in different species (Mitchell et al. 2008; Schmelting et al. 2008, 2009; Meyer et al. 2010; Adler et al. 2013; Martel et al. 2013). HDO measures blood pressure in a rapid, stress-free manner even in freely roaming animals (Schmelting 2010), and if cardiovascular instability is present, it further adds valuable information on SV, SVV, SVR (Egner et al. 2013) and dysrhythmia. HDO conforms with FDA requirements so it can be used in large-scale GLP studies in a variety of different animal species such as dogs, cats, rabbits, rats as well as NHPs (in particular marmosets, cynomolgus, rhesus, etc.), though it can be adapted for use in almost any species.

1 Introduction

It is vital to determine blood pressure (BP) in order to avoid any potentially life-threatening impact of a drug on this parameter. In safety pharmacology (SP) and toxicology (Tox), it is especially important to reliably test a large number of individual animals to guarantee drug safety in this respect. If a drug is developed to affect blood pressure, it is vital to monitor the efficacy of that impact. Since a large number of animals often need to be tested, a fast, affordable and easy-to-use technology is required. This means that implanted systems are inadequate in such a scenario. Although the importance of monitoring blood pressure and other cardiovascular parameters in SP and Tox studies is undisputed (e.g. Markert et al. 2007; Taylor et al. 2007; Pugsley et al. 2008), the limitations of conventional non-invasive methods (Stepien et al. 2003; Brown et al. 2007) limited determination of blood pressure in such studies.

The dog is a commonly used species for the evaluation of cardiovascular parameters including blood pressure and is recommended by the ICHS7A guidance (US FDA 2001) for safety pharmacology studies on human pharmaceuticals. This

guidance states the preference for in vivo studies using experimental models with conscious animals over models with anesthetised animals. This is indicated because anaesthetics can have a more or less pronounced impact on the cardiovascular system. This can mask drug-induced effects or alternatively exaggerate them due to marked suppression of the baroreceptor reflex. Studies in conscious dogs are also susceptible to physiological mechanisms. The animals are capable of reacting to their environment, especially the sudden onset of (unknown/unexpected) noises. Excessive movement of the animal during the measurement is also possible. The 'white coat effect' (Brown et al. 2007; Egner 2007) can also play a role. This does not however limit the implementation of BP monitoring in SP and Tox studies as long as an appropriate technology is used, which allows real-time analysis of the incoming pulse waves, differentiation of pulse waves from artefacts and thus a reliable signal analysis, allowing verification of true blood pressure as it is secured with HDO.

2 Accuracy of the HDO Technology

2.1 Conventional Non-invasive Systems

Conventional non-invasive BP technology (conventional oscillometry like Sharn/Cardell, Surgivet BP monitor, Doppler technology, etc.) has clear limitations in different respects:

- Deflation rate is not adjustable: in conventional oscillometric devices, deflation rate is fixed at 3 mmHg/s. This is appropriate in humans with a heart rate of 60 beats per minute (bpm), since according to the AAMI in human medicine, a non-invasive technology should pump up the cuff pressure to 180 mmHg and then deflate it completely within 60 s. Thus, 180/60 = 3 mmHg/s, allowing it to detect every pulse wave. In animals, however, heart rates often differ dramatically from human heart rates. Beagle dogs generally have a heart rate of 80–120 bpm, conscious cats easily reach between 160 and 250 bpm and marmoset monkeys even 400–600 bpm. This clearly indicates that a constant deflation rate of 3 mmHg/s is not sufficient or appropriate for all species. With an inappropriate deflation rate, a substantial number of pulse waves will be missed in all smaller species, like beagle dogs, but even more significantly in cats, cynomolgus, common marmosets, rabbits, rats and many other rodent and non-rodent species.
- Linearity: All blood pressure device valves available on the market offer limited linearity, meaning that—depending on the manufacturer—they do not have specifications for pressures above 160–170 mmHg or below 60–80 mmHg. This limits accuracy to the range of 60–170 mmHg, primarily due to (a) valve limitations and (b) the overall algorithm. In order to overcome these limitations and to ensure accuracy at all pressure situations (0–300 mmHg), it is mandatory to sample and analyse incoming signals and use the data to program the valves

towards linearity in all pressure ranges. Conventional oscillometric and Doppler devices are too slow to allow for valve programming in real time (conventional oscillometry works with 8 bit processors, Doppler is manually operated, thus is much slower). Real-time programming requires a minimum processor speed of 32 bit.

– Algorithm: Conventional oscillometric devices need to match incoming signals to a preprogrammed shape of an ideal pulse wave because of their slow processors. The preprogrammed pulse wave is a human pulse wave, which differs substantially from the incoming signals of an animal with its high heart rates, lower cardiac output (compared to humans) and smaller arterial diameter. Conventional oscillometric units also generally cannot measure all three pressures and usually measure (depending on the manufacturer) mean or mean and systolic pressure. Diastolic or both systolic and diastolic pressure are often calculated with an algorithm. This obviously can be a more or less dramatic source of inaccuracy. Since not only mean arterial pressure but also artefacts produce very strong signals, such units can be misleading and consequently measure an artefact and not the blood pressure.
– No visual control of pulse wave and pulse wave-related analysis

These limitations clearly limit the use of conventional NIBP monitors in research settings.

2.2 High-Definition Oscillometry

High-Definition Oscillometry (HDO) is a patented technology which has been developed with the latest in hardware components such as a 32 bit processor, high-end sensors and specific cuff material to avoid resistance. This allows for very sensitive and much faster analysis of incoming signals and even real-time assessment of the pulse wave with real-time programming of the valve to achieve linearity over a pressure range of 0–450 mmHg in vitro. In animal models, maximal pressures of 297 mmHg SAP and minimal pressures of 18 mmHg DAP have been provoked and are correctly detected by HDO (Baumgartner et al. 2009). Values have been compared to different direct systems such as the HSE in dogs and rabbits, DSI in dogs and cats, marmosets and cynomolgus monkeys as well as others (see Sect. 2.2.2). HDO also uses a newly developed software, adding to high accuracy and differentiation of artefacts from pulse waves. This is true in regular rhythms but also in arrhythmias which clearly affect beat-to-beat cardiac output as well as in situations with excessive peripheral vasoconstriction.

2.2.1 HDO Features

HDO science units offer a pulse detection of up to 600 bpm, allowing for accurate readings even in small laboratory animals like common marmosets, rabbits, rats, etc. During the first reading, HDO analyses the pulse waves of the animal and then automatically adjusts deflation rate for subsequent readings based on this data. This

pulse-adapted deflation rate results in fast readings with fast pulse rates. Thus, animals with a pulse rate above 250 bpm can be measured in as little as 8 s. The speed of measurement further adds to convenience of using this system in a research setting, where speed and repeatability play an important role in addition to accuracy. Additional analysis is directed towards information reflecting cardiac output and arterial diameter, resulting in different signal strength and finally leading to an adjustment of gain to secure readings even in severely affected situations like shock or other reasons for massive peripheral vasoconstriction as well as severely impaired cardiac output. Further pulse pressure ($PP = P_{systolic} - P_{diastolic}$) can be reliably determined as HDO measures both systolic and diastolic blood pressure, adding on information about arterial compliance. On-screen real-time visualisation allows the quality of a reading to be judged while it is taken. Artefacts, rhythm and arrhythmia are further visualised. To definitively determine the rhythm, an ECG is recommended (Fig. 1a–c).

Raw data security, filing/print out options and different level rights according to the audit trail are given at any stage of a study and can be customised to specific needs or requirements.

2.2.2 HDO Accuracy and Reliability

In order to evaluate HDO, this new technique was compared to different invasive techniques. Invasive measurements are considered the 'gold standard' in veterinary medicine. So far, dogs (conscious and anesthetised), cats (conscious), rabbits (anesthetised), common marmosets (conscious), cynomolgus monkeys (conscious and anesthetised), ferrets (anesthetised), minipigs and horses have been evaluated with accuracy and precision of use having been shown in these species. Some of these studies have been presented as scientific abstracts or poster and published respectively (Caulkett et al. 1998; Schlumbohm et al. 2006; Baumgartner et al. 2009; Schmelting et al. 2008, 2009; Mitchell et al. 2008; Greiter-Wilke 2009; Meyer et al. 2010; Martel et al. 2013; Egner et al. 2013).

In summary, the results demonstrated good correlation, high precision and repeatability.

In a study using anesthetised dogs (see Baumgartner et al. 2009), direct measurement (using a HSE, Millar Catheter 3 French SPR249) was compared to HDO (S + BmedVET, Babenhausen; HDO Science 1.36.02 hardware and 1.7.5.2 software version). The best agreement between the two methods was found to be SAP, since HSE was not displaying the mean. Within single animals, a significant correlation between the measurement methods for SAP was observed (correlation coefficient $r = 0.895$; $p < 0.001$). The mean difference between the two methods was 0.18 ± 9.15 mmHg ($p = 0.632$, see Fig. 2a). Also for DAP, a significant correlation between the two methods was found ($r = 0.875$; $p < 0.001$) with a mean difference of 5.6 ± 7.3 mmHg ($p < 0.001$, see Fig. 2b).

In this study, blood pressure was manipulated with various drugs in order to reach very high and very low pressures with and without arrhythmias. The highest correlation and lowest bias was reached in non-arrhythmic situations (see Fig. 3).

Schmelting et al. (2008) compared data in conscious and anesthetised cynomolgus and found excellent correlation of pulse and mean arterial pressure

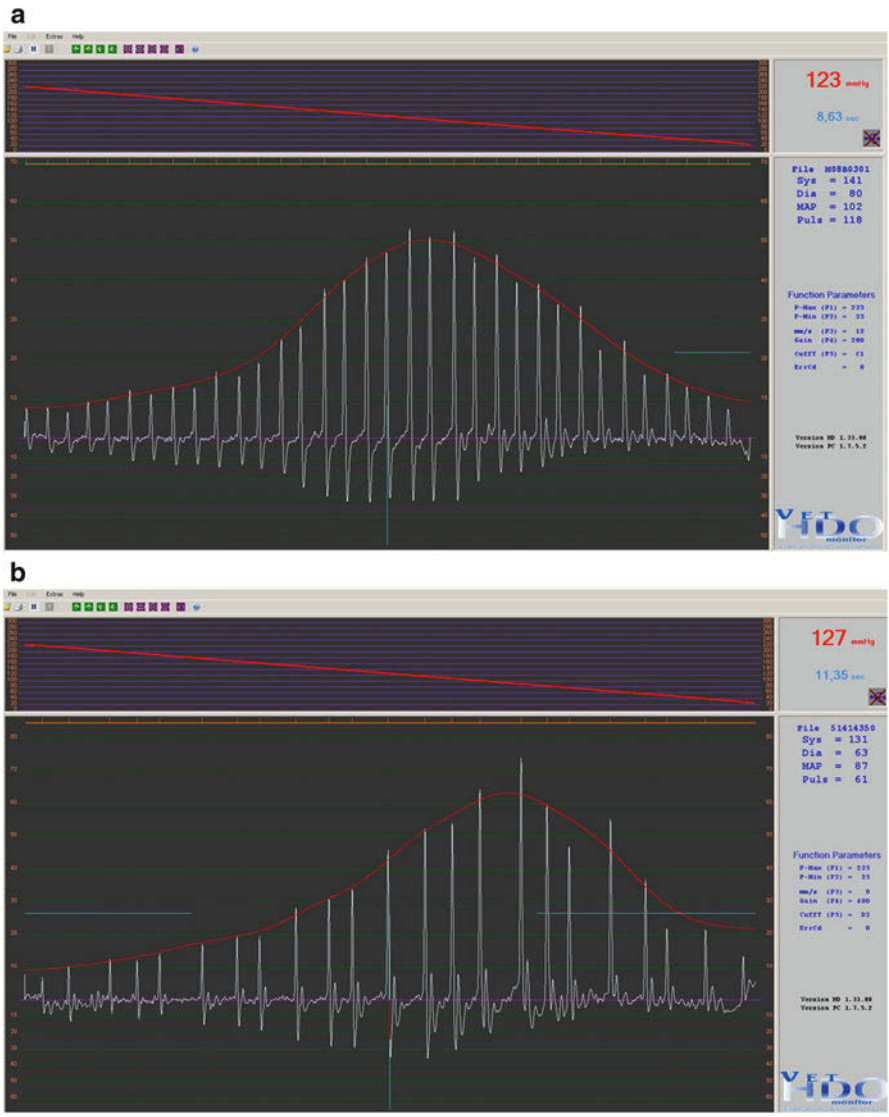

Fig. 1 (continued)

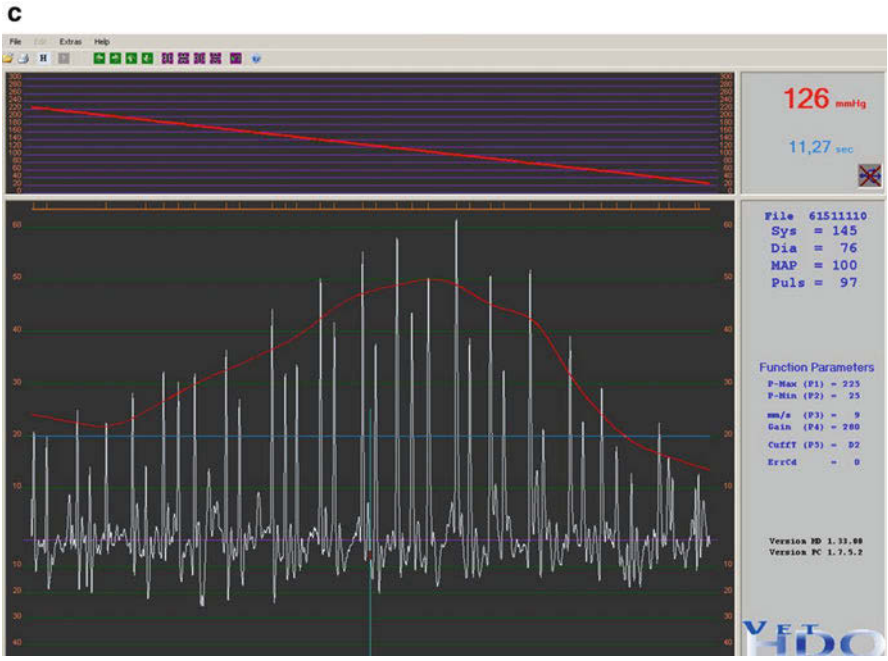

Fig. 1 (**a**) A normal HDO curve of a complete reading. *From the right*: pre-systolic amplitudes followed—with continuous deflation of the cuff—by the opening of the artery (i.e. *bell shape*). Due to initially turbulent flow, amplitudes become continuously greater up to a maximum: mean arterial pressure. At this stage, central laminar flow can be found resulting in decreasing amplitude height. (**b**) An HDO curve in a dog with respiratory sinus-arrhythmia. (**c**) An HDO curve with sinus node block. Note the impact of the arrhythmia on stroke volume (SV) due to prolonged diastolic filling and subsequent increase in contraction observed due to the Frank–Starling mechanism

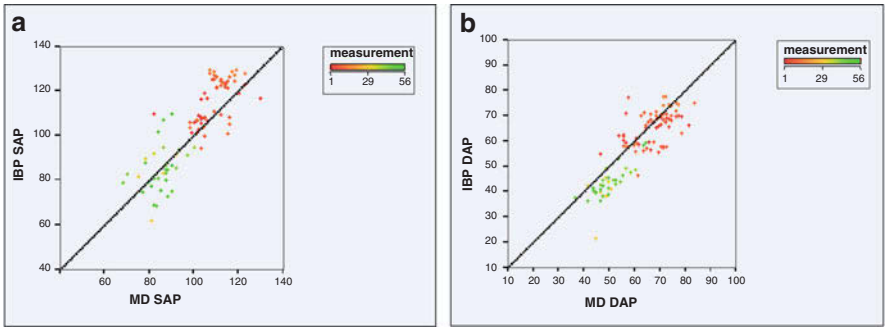

Fig. 2 Bias for systolic (**a**) and diastolic (**b**) arterial pressure (see Baumgartner et al. 2009)

throughout the trial. The investigators even suggested that HDO may be regarded as an alternative for implanted systems especially to be used in animal models for

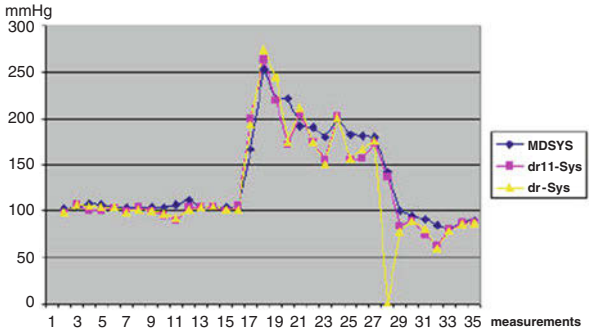

Fig. 3 HDO results vs. the final, and the average of 11 HSE readings, during an HDO measurement interval (Baumgartner et al. 2009)

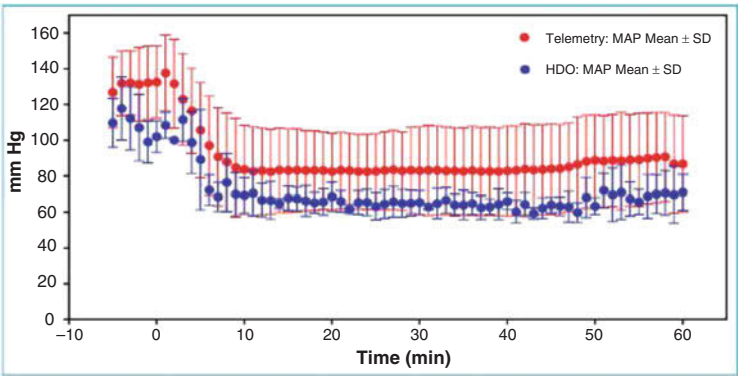

Fig. 4 Mean arterial pressure measured in conscious and sedated male cynomolgus monkeys with telemetry and HDO. The intra-individual variances are less prominent with HDO compared to telemetry indicative of higher precision (Schmelting et al. 2008)

cardiovascular research. As the implanted system was located more centrally and HDO at a peripheral artery, mean arterial pressure measured higher with the implanted system, but the difference to the measurements taken with HDO was equal. That was expected due to the different arterial wall layers in the different types of arteries. Even more, the intra-individual readings show a higher precision in the HDO group compared to the direct group (Fig. 4).

In a study with paced rabbits (unpublished) under anaesthesia, the animals finally went into atrial fibrillation with a mean arterial pressure as low as 34 mmHg before they died. In this very low pressure situation, HDO still measured SAP, DAP and mean pressure with mean correlating ±1 mmHg with directly obtained mean pressure, whereas the direct system could no longer determine SAP and DAP.

Meyer et al. (2010) compared the ability to detect Torcetrapib-mediated effects on blood pressure and heart rate when measured by both HDO and telemetry (DSI)

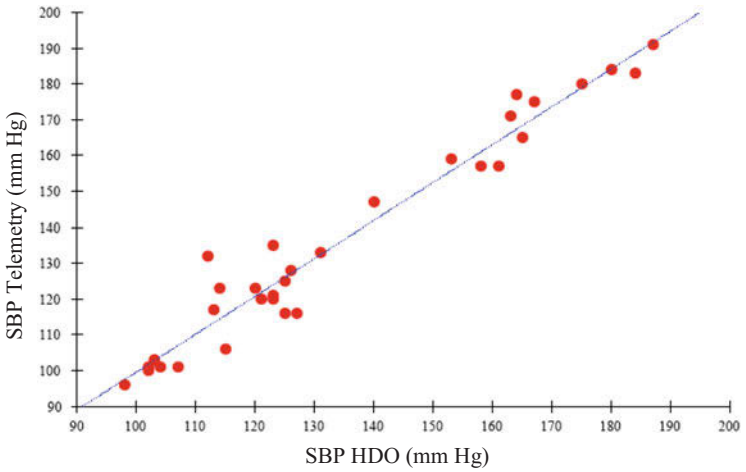

Fig. 5 The linear regression between SBP and values measured using either HDO or telemetry systems in an individual animal (cat N° 2008024). Note that the SBP correlation coefficient was $r = 0.98$ (Martel et al. 2013)

and found that HDO detected a 15.8 ± 10.4 mmHg increase in MAP compared to telemetry and detected a virtually identical 15.8 ± 5.3 mmHg increase with an identical heart rate. They summarised that HDO provides a useful and accurate method for non-invasive blood pressure measurements.

Martel et al. (2013) also compared HDO to a DSI implanted system and challenged blood pressure pharmacologically to reach hypotensive and hypertensive situations. SAP showed a mean correlation coefficient of 0.92 ± 0.02 with individual correlations as high as 0.98 (Fig. 5) and for DAP 0.81 ± 0.02. The slightly lower correlation for DAP was discussed as being due to the difference in arterial wall structure in more centrally versus peripheral arteries.

These differences have been described by other authors and in different species as well (see Bramlage et al. 2009; Safar and Jankowski 2009; Mitchell et al. 2010). In this study, ACVIM (American College of Veterinary Internal Medicine) requirements have been fulfilled for the first time. The authors conclude that this study highlights 'HDO is the first and only validated non-invasive blood pressure device and, as such, it is the only non-invasive reference technique that should be used in future validation studies'.

Mietsch and Einspanier (2014) assessed the applicability of HDO for use in the common marmoset monkey and from their study defined the best location for measurement: the hind limb. Their study supports the fact that HDO values enable for a reliable comparison of blood pressure parameters in cardiovascular, toxicological and metabolic research. Similar statements were given by Bramlage et al. (2009) for the common marmoset and Niehoff et al. (2014) for the cynomolgus monkey. In both cases, data have been compared to telemetry methods.

2.3 GLP and FDA Requirements

In order to use HDO and be compliant with Title 21 CFR Part 11 for GLP studies, raw data security and a closed system is mandatory. HDO science is a closed system containing a laptop/netbook with pre-installed software + HDO unit along with the relevant cuff(s). The software is customised according to the in-house requirements of each facility and in compliance with FDA for GLP required studies. The unit is attached to the laptop/netbook via a USB adapter (or Bluetooth wireless). The unit and laptop/netbook are one entity. A server solution is possible too. The HDO science units work with clear right classes according to an agreed audit trail, ensuring raw data security, interim study analysis during each stage of a study and in each right class according to the assigned user rights. Animals can be selected for use in each study and monitored in terms of any kind of reaction, behaviour, disease, side effect, etc., during the study (Fig. 6). Single sections of data can be selected and analysed at each stage and in each right class (password secured) according to the rights contained within the audit trail. An electronic fingerprint system is also available to close a study and produce a backup file.

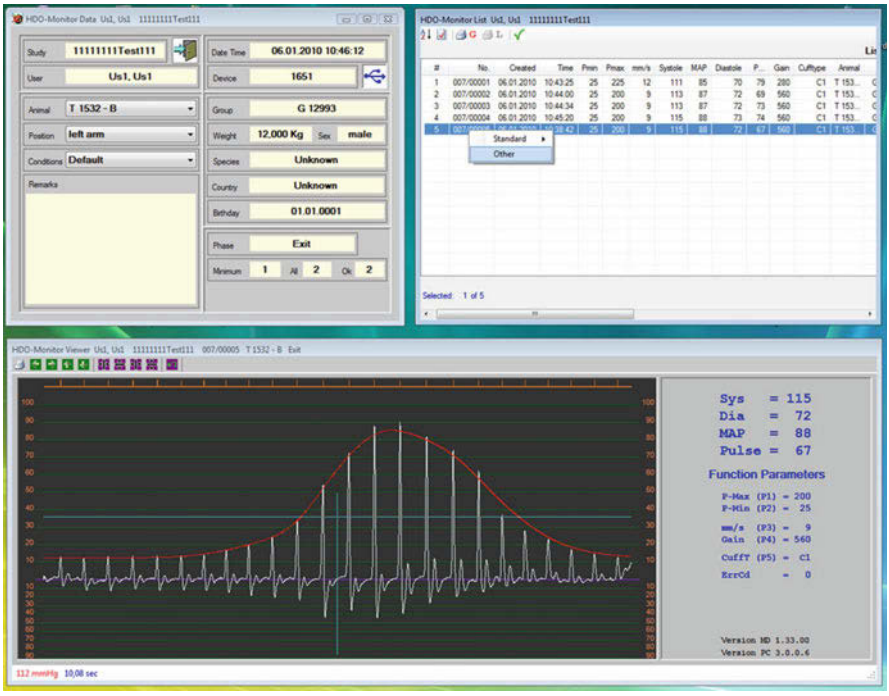

Fig. 6 A screenshot of the HDO science user window

2.4 HDO in Safety Pharmacology (SP) and Toxicology (Tox) Studies

Due to the ease of use, attained fast measurements and the accuracy and reliability of HDO, this system is suitable for use in large animal numbers and thus for SP and Tox studies.

2.4.1 Training of Personnel on the Technique

Training of the personnel/technicians can be successfully achieved within 1–2 days with a high level of understanding in terms of (a) how to obtain and judge, (b) what is an actual good reading, (c) how to avoid artefacts and (d) how to secure the relevant numbers of acceptable readings for a study being achieved.

2.4.2 The Use of HDO in Non-clinical Research

How to Use HDO in the Conduct of a Study

Due to the fact that HDO is a highly sensitive methodology, measurements can be taken easily on small peripheral arteries like at the tail. Alternatively, measurements are possible on the front limb or the hind limb. Different cuff sizes and material are available to secure accurate readings. The system is set up to the specific species it will be used for in order to avoid user or handling errors. Animals should be brought in and maintained in a relaxed position. Cuff placement should be made at the same height as the base of the heart to secure reliable readings (see Fig. 7). If the cuff position is higher than the base of the heart, the readings are falsely low, and if it is placed below the base of the heart, the readings will be falsely high. If such errors are made the difference can be as much as 0.8 mmHg/cm. If measurements have to be accepted, e.g. in a sitting position on the tail, the height difference between that position and the base of the heart needs to be considered in the data analysis, e.g. if the height difference is 25 cm, the pressure difference is 20 mmHg (or 25 cm $\times$ 0.8 mmHg/cm).

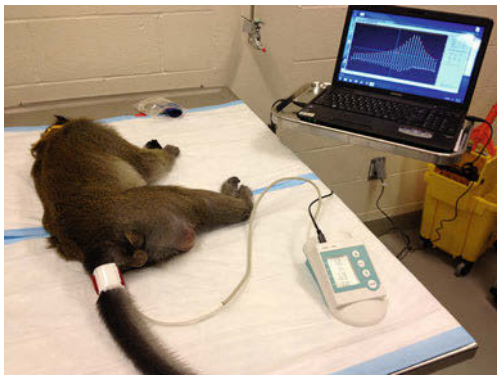

Fig. 7 A cynomolgus monkey with a cuff placed at the base of the tail. An excellent measurement can be observed on the computer screen without any artefact

Fitting of the HDO Cuff

The cuff should be fitted snugly, but not too tight, in order to allow the bladder to inflate correctly. The general rule is that if the tip of the experimenter's small finger just fits in between the skin and the cuff, the fitting is placed correctly (see Fig. 8). However, in case the cuff is placed too loosely, the obtained HDO graph appears to 'drop' at the end, indicating to the investigator that the cuff should be closed and made snugger. If the cuff fitting is too tight, there is either no measurement detected or an undulating HDO graph is observed, indicating a nonvalid measurement.

After placement, the relevant cuff is then inserted into the fast connector/disconnector and the unit is started. The system then calibrates itself (each time it is started) which takes a few seconds but is then ready to for use in measuring BP (see Fig. 7). Note that GLP calibrations however need to be scheduled appropriately.

Restraint, when necessary, should be selected according to (a) what are the animals used to and (b) what is best tolerated. Quite often some animals like Cynomolgus monkeys and rats are much more relaxed in a tube (see Fig. 9a). Common marmosets often do great in a chair. Dogs seem to prefer to stand but can be placed in a sling as well (Fig. 10c), while cats prefer to lie on the lap of the technician. Such preferred positions for each species can be recorded in the animal data files for future studies, making the planning of such studies much easier.

If measurements without restraint are needed, HDO can be set up as a 24-h monitoring system, transferring data by Bluetooth to the recording computer. For this type of study, the cuff is place on the tail and the unit is put into a jacket. HDO measurements are automatically initiated but can be additionally started any time from a computer anywhere within 1,000 m from the animal. This methodology and capability mimics telemetric measurements. The limitation of such a study design is that acquired HDO measurements will likely have significant amounts of artefacts, especially if the animal is moving at the moment the measurement is made. However, Schmelting (2010) have shown that sufficient excellent readings can be achieved using HDO as an ambulatory Holter system, which supports the use of this technology on freely roaming animals.

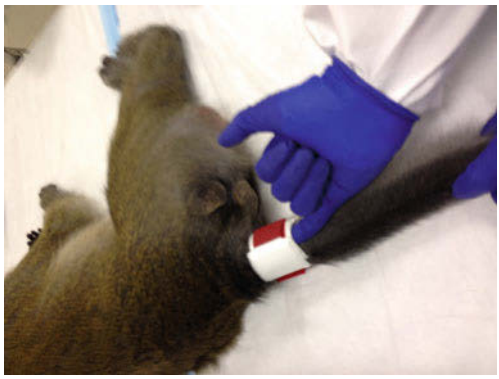

Fig. 8 Demonstrates the fitting test for cuff placement using the small finger tip of the experimenter

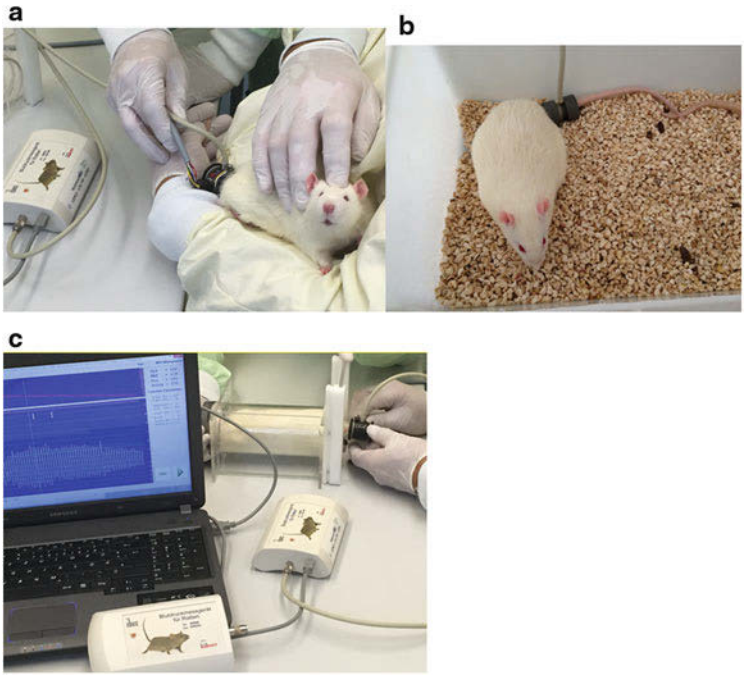

Fig. 9 This figure shows that HDO measurements made in the rat are possible when either minimally restrained (**a**), freely roaming (**b**) or while in a tube restrainer (**c**). Note that the restrainer-derived HDO measurements do not appear stressful and result in measurement of excellent traces, as shown on the monitor (**c**). Note that freely roaming rats tend to move during recording of the measurements creating possibly too many artefacts for analysis

Once the cuff is in position, the first reading can be initiated. While the measurement takes place, the generated curve should be evaluated by the investigator. Each heart beat creates a pulse wave which can be seen in real time on the screen (Fig. 11a). Artefacts are easy to detect, and in such a case, the technician can immediately investigate the source of such artefacts in order to avoid it for subsequent readings (Fig. 11b).

Some of the most frequent sources of artefacts in HDO measurements include:

- Active movement of a limb (muscle tension)
- Shivering
- Laboured breathing
- Pressure on the cuff from outside (i.e. a person touching the cuff, the animal waking with the tail and bouncing the cuff against a surface, etc.)
- The restraint tube bounces against something

HDO Data Analysis

The science software allows for fast on-screen analysis of data, evaluation, selection and closing of a level/study. Detailed information on blood pressure, pressure

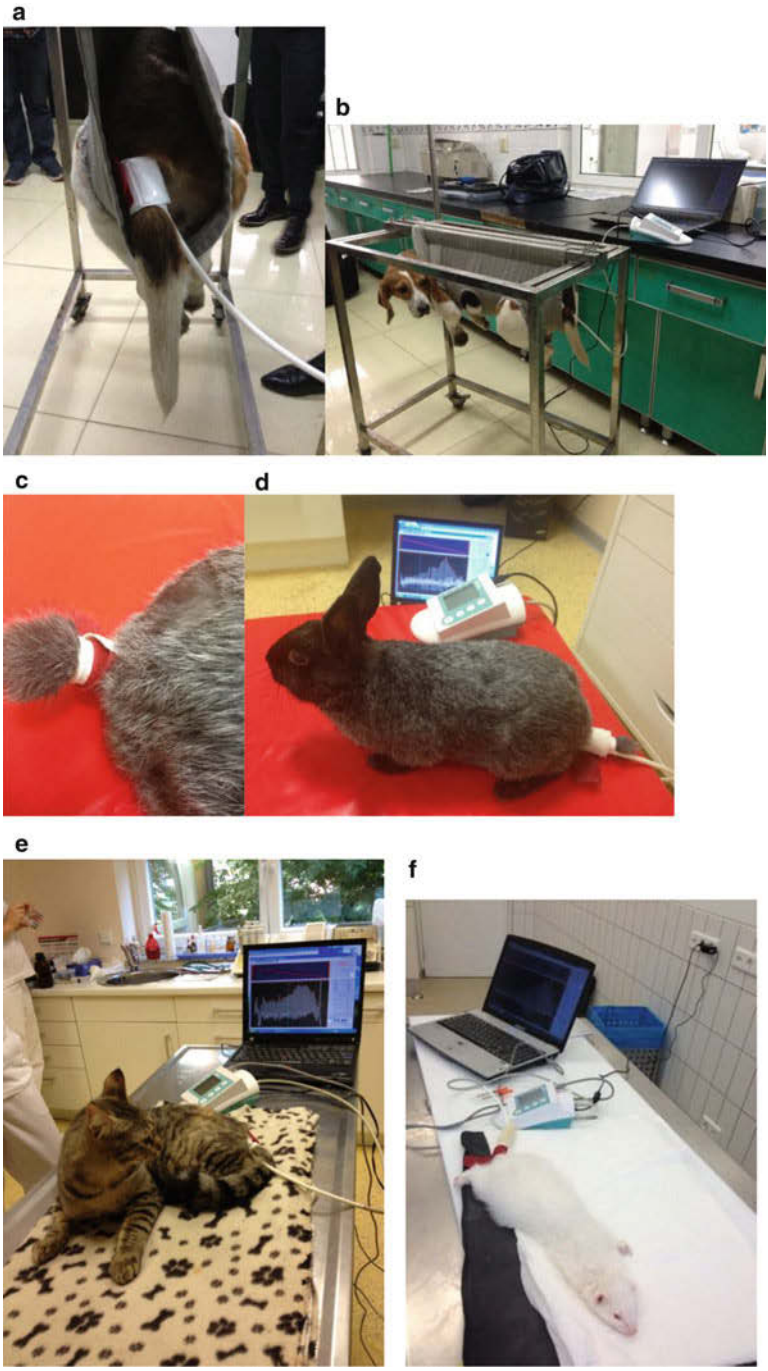

Fig. 10 Examples of HDO tail cuff measurements in different animal species including the dog (**a**, **b**), the rabbit (**c**, **d**), the cat (**e**) and the ferret (**f**)

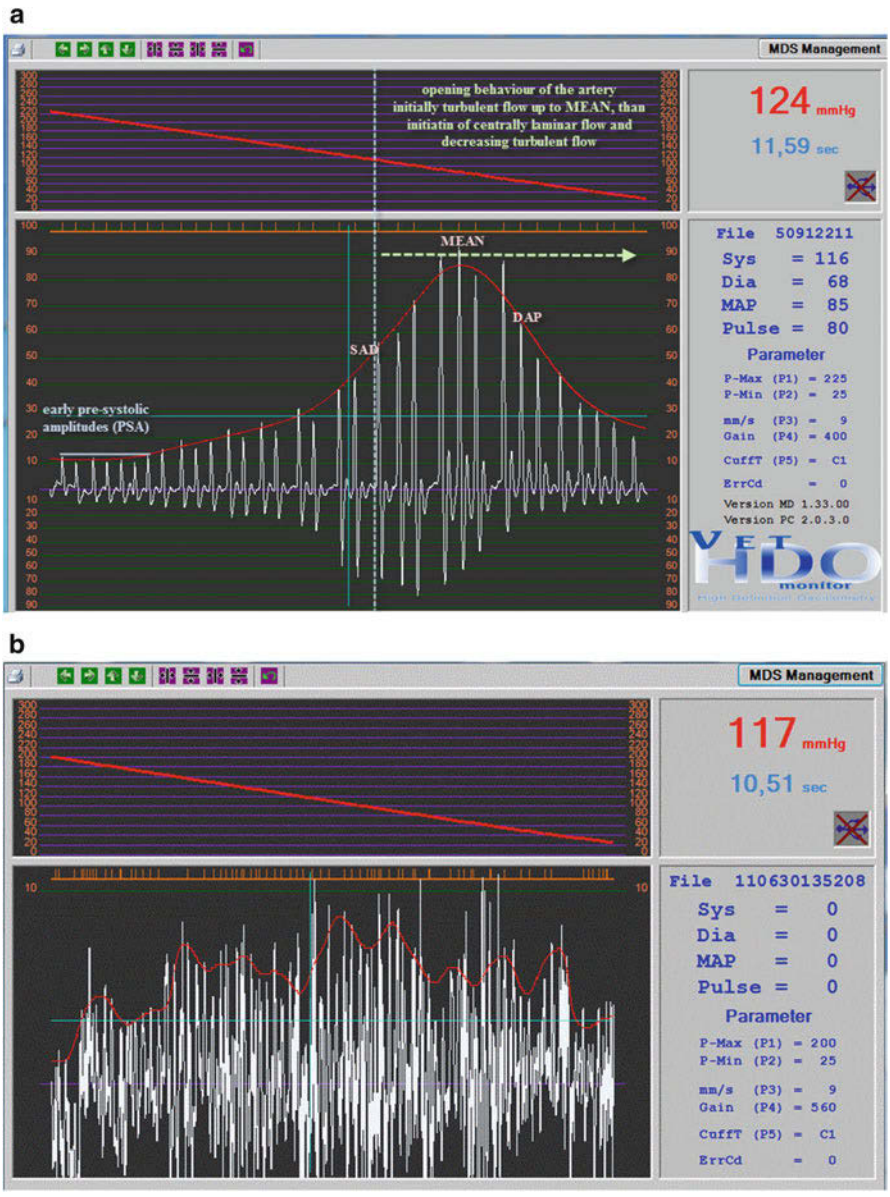

Fig. 11 (**a**) An example of an excellent HDO reading. Note the linear deflation line (*red*) that indicates no artefact. Pulse wave analysis (PWA) information is detailed in the comments. (**b**) Measurements taken from the front limb of a cat. Note in the *upper panel* that while deflation remains linear, artefacts are clearly visible in the *lower panel* (due to shivering and heavy breathing). Watching the animal while the events appear helps to identify the source of the artefact and avoid it for following readings (note that cuff placement on the tail avoid shivering and breathing artefacts)

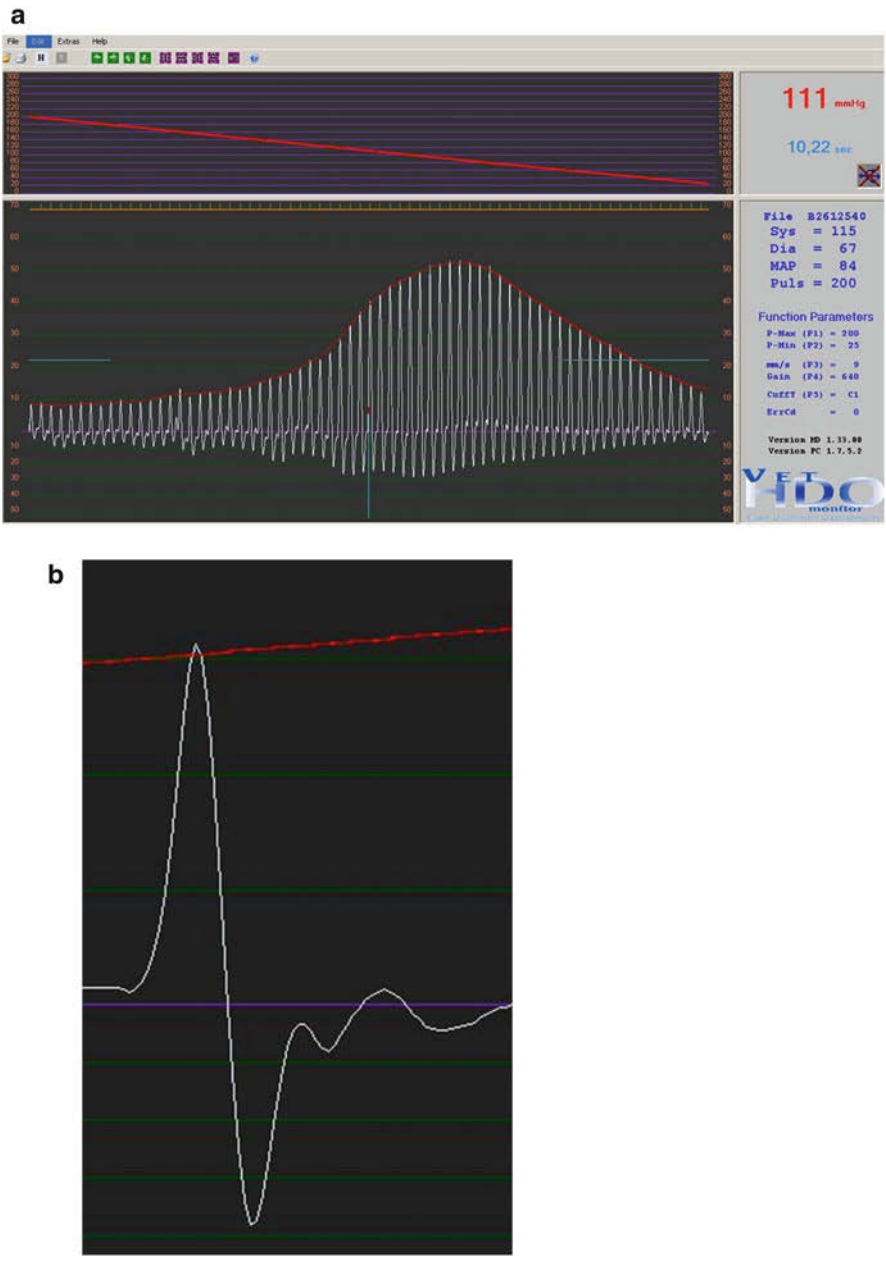

Fig. 12 (continued)

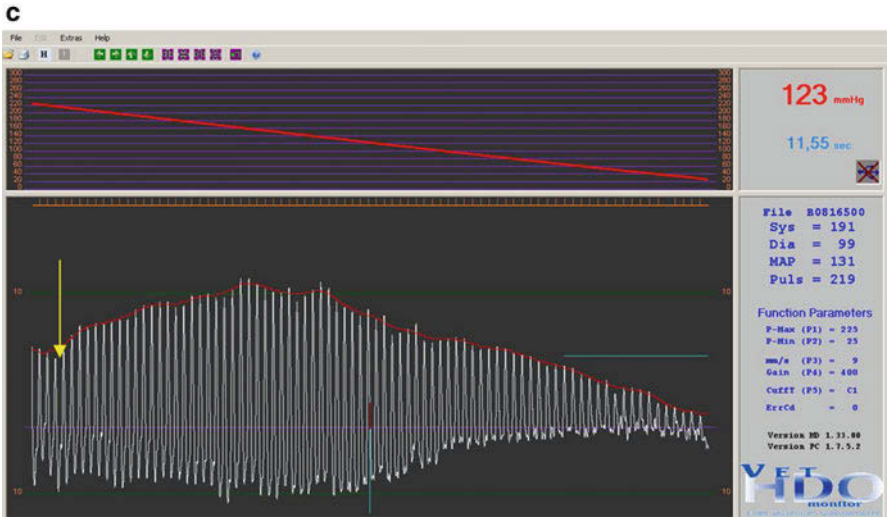

Fig. 12 (**a**) HDO analysis of a complete reading. Normal arterial opening behaviour observed, low pre-systolic amplitudes (i.e. low PSA = good arterial compliance), constant increase and decrease of amplitude height during opening of the vessel (i.e. stable cardiac output), spaces between single amplitudes are equal so a rhythmic situation exists (i.e. no arrhythmia). (**b**) Single wave analyses can be conducted at an increased scale from the HDO measurement in (**a**). (**c**) An example of a cat with chronic kidney disease and hypertension: high pre-systolic amplitudes (PSA) (*arrow*) indicate impaired arterial compliance

wave analysis and overall analysis of the arterial opening behaviour of the vessel and related information are easy to obtain (see Fig. 12a–c for examples).

Additionally, stroke volume (SV) or stroke volume variance (SVV) in particular, in conjunction with arrhythmias, can be visualised and subsequently analysed (Fig. 13).

HDO pulse wave analysis (PWA) has been compared to the Picco, Lithco and Vigilance invasive measurements of SVR, SV, SVV and cardiac output (Egner et al. 2013). Blood pressure, systemic vascular resistance and cardiac output were altered pharmacologically to identify the sensitivity of HDO compared to the gold standard invasive technologies. As demonstrated below, SVR measured with HDO follows the same trend as the invasive method. As HDO was measured more peripherally than the Picco system, additional regulative response of the periphery in comparison to the more central response can be identified. This shows nicely that SVR can be evaluated with HDO, making this technique particularly useful in cardiovascular and metabolic research (Fig. 14).

HDO and Pulse Transit Time Measurement: The Next Generation

HDO can be upgraded with a specific cuff. The extra features of this cuff (see Fig. 15a, b) allow for an additional optic blood flow evaluation and further pulse transit time (PTT) measurements while evaluating blood pressure and PWA. This cuff has three sensors that allow for accurate investigation of the PTT leading to additional information regarding arterial stiffness and how drugs may affect this

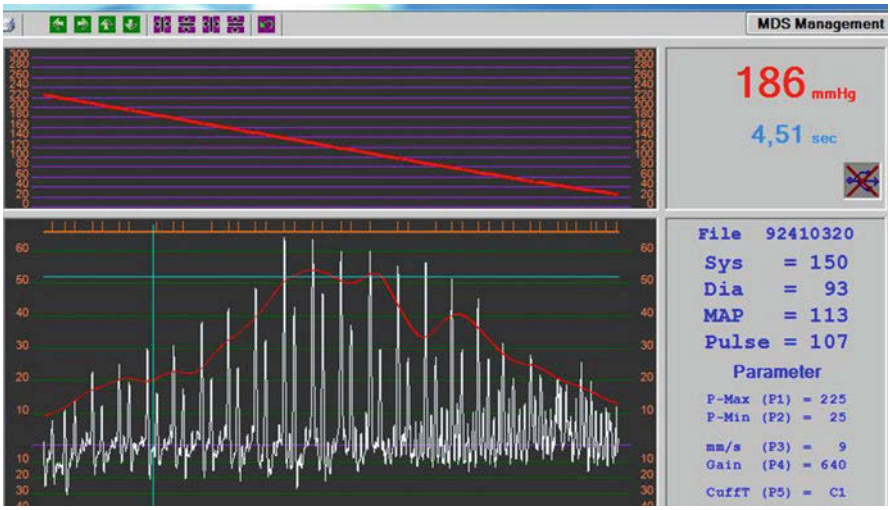

Fig. 13 This figure shows the clear impact of altered beat by beat stroke volume (SV) (visualised as the height of the single amplitude) due to the presence of a bigeminal rhythm in the animal

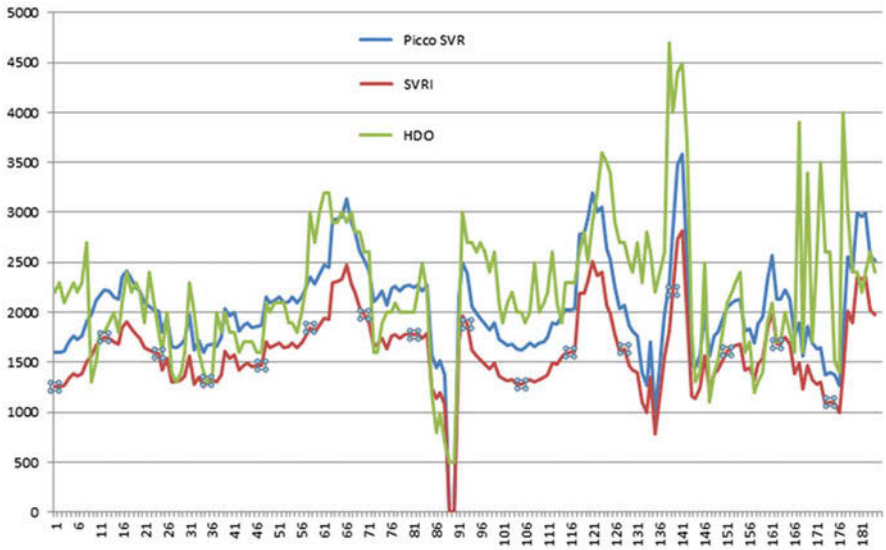

Fig. 14 A comparison of systemic vascular resistance (SVR) measured using the HDO and Picco systems after a challenge with drugs (see Egner et al. (2013) for details)

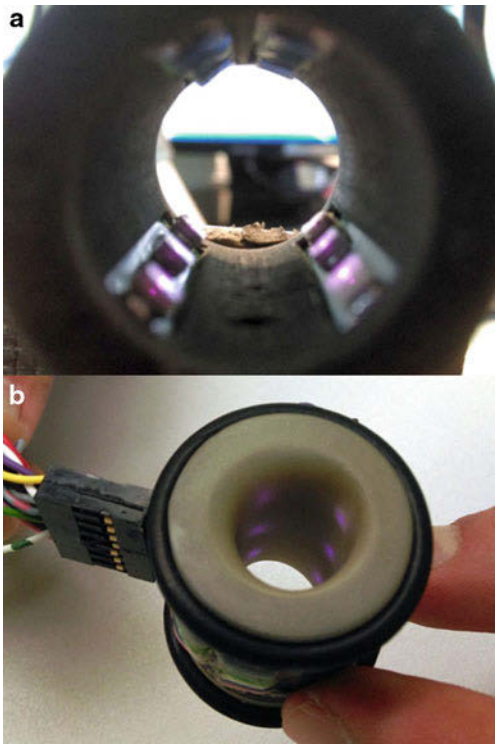

Fig. 15 (**a**, **b**) Use of a special cuff with photo sensors for PTT measurement

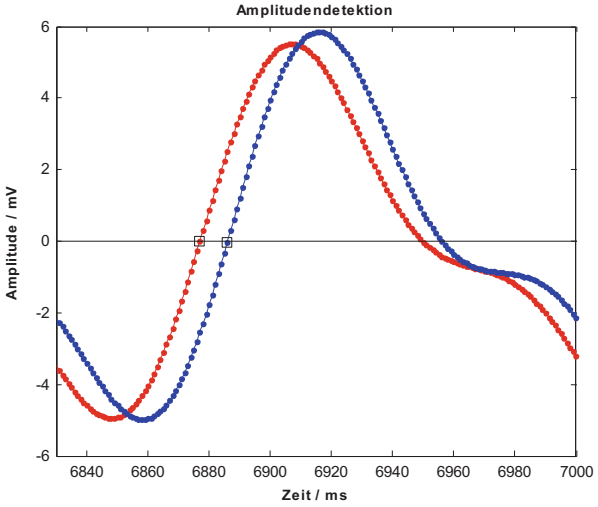

Fig. 16 Photosensor measurements made with an optical cuff. While sensor 1 and 3 are active for the reading, sensor 2 (in the *middle*) is only used to stabilise the system. The two curves that are obtained are displayed on the screen as above and allow the investigator to read PTT easily. Values are automatically displayed and stored

parameter which will further support cardiovascular and metabolic research (Fig. 16).

3 Limitations of HDO

When using the HDO technique on conscious animals, physiological stress may play a role in modulating the determination of blood pressure in studies, especially when the animals need to be handled. Thus, proper training of the personnel is required, and implementation of a strict protocol for data acquisition is mandatory in order to avoid excitement. As animals seem to react either initially or with time, it makes sense to evaluate the group upfront and include this criterion into the individual animal data sheet. This allows for the subsequent selection of animals with the same type of behaviour. With this in mind, studies with HDO can be planned with large numbers of conscious animals without running out of time limits. Note that any handling-induced excitement does not play a role if HDO is used as an ambulatory Holter system.

4 Summary

HDO is a reliable and accurate method for non-invasive blood pressure measurement. The use of this technology allows for blood pressure and cardiovascular information to be more frequently assessed and thus included into safety pharmacology and toxicology studies. Early detection of impaired vascular resistance can be added as a key parameter in the detection and assessment of heart and kidney disease as well as for use in metabolic research such as diabetes. Visualisation and analysis of single pulse waves, pulse pressure, the opening behaviour of the artery and in particular also pulse transit time might open new dimensions in the overall cardiovascular and metabolic evaluation of drugs for use in patients.

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Part III

Supplemental Safety Pharmacology

The Safety Pharmacology of Auditory Function

Matthew M. Abernathy

Contents

Abstract

Safety pharmacology satisfies a key requirement in the process of drug development. Safety pharmacology studies are required to assess the impact of a new

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chemical entity (NCE) or biotechnology-derived product for human use on vital systems, such as those subserving auditory function. Safety pharmacology studies accordingly are defined as those studies that investigate the potential undesirable effects of a substance on auditory functions in relation to exposure in and above the therapeutic range. Auditory safety studies should be designed with the primary objective of determining how administration of a compound influences normal hearing. If an effect on hearing is identified, then it is necessary to determine through histopathology the underlying mechanism for the observed hearing loss. Since the auditory system contains a heterogeneous mixture of structural and cellular components that are organized in a very complex and integrated manner, it is necessary to clearly identify the underlying primary mechanism or target of the new chemical entity that produced the hearing loss. This chapter will highlight major components of auditory function with regard to potential opportunities for drug interaction. Aspects of designing ototoxicity studies will be discussed with an emphasis on standards deemed necessary by the US Food and Drug Administration. Additionally, classes of ototoxic compounds and their proposed mechanisms of action are described in depth.

Keywords
Auditory function • Drug safety • Otic microscopy • Auditory brainstem response evaluations • Cytocochleogram • Intra-aural drug administration

1 Auditory Physiology

Hearing is a complex function which involves the transduction of mechanical sound pressure wave stimuli into neuronal electrochemical signals. The many structures of the auditory system work together to amplify sounds and convert mechanical energy to produce robust neural signals. Further, the auditory system enables selective bandwidths to be targeted and fine-tuned which permits greater sensitivity and discriminative function between frequencies. This critical element of hearing enhances the ability of an organism to survive in its environment. Understanding how the many structures of the external, middle, and inner ear function apart, and as a whole, is essential for evaluating drug-induced hearing loss.

Hearing begins with the external ear, the pinna, which, with its many folds and ridges, is able to locate and capture sound waves from the immediate environment. The generalized conical shape of the external ear meatus funnels sound waves downward into the auditory canal where the sound pressure waveform then interacts with the tympanic membrane, also known as the tympanum. Similar to a stick hitting a drumhead, the sound wave impacting the external side of the tympanic membrane begins a vibration equivalent to the frequency of the original sound pressure wave that initiated it. On the opposite or internal side of the

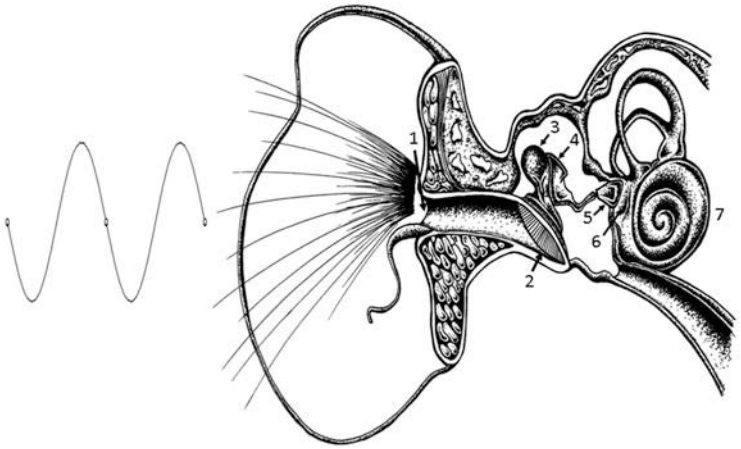

Fig. 1 Anatomy of the external, middle, and inner ear of a guinea pig. Major structures are as follows: *1* external auditory canal, *2* tympanum, *3* malleus, *4* incus, *5* stapes, *6* round window, and *7* cochlea

tympanic membrane is the middle ear cavity (refer to Fig. 1). The vibration of the tympanic membrane sets into motion a set of small bone-like structures referred to as the middle ear ossicles. The ossicles are actually a chain of three bones, the malleus, incus, and stapes (commonly referred to as the hammer, anvil, and stirrup, respectively). These small bones vibrate in harmony with the direct stimulation from the tympanic membrane. The ossicles are supported by two small muscles: the stapedius muscle attaches to the stapes, and the tensor tympani muscle attaches to the malleus, both of which act to dampen loud noises and protect the inner ear from acoustic trauma. It is said that movement of the tympanic membrane the width of a single hydrogen atom has the capacity to initiate downstream cellular actions in the inner ear. The structure, function, and sensitivity of the auditory system remain a marvel to modern science.

The cochlea is next in the series of structures that operate to convert sound wave energy from the environment to an electrochemical message that can be processed by the central nervous system. The cochlea is an intricate, fluid-filled structure consisting of multiple specialized membranes and the cellular organ of Corti. The foot of the bony ossicles in the middle ear mechanically strikes another "drumhead" located on the fluid-filled cochlea, called the oval window. The footplate of the stapes rests upon the oval window, and mechanical sound pressure vibrations within the ossicles produce rhythmic depression of this membrane. The pulsatile movement of the oval window forces the fluid within the cochlea to move in synchrony, forming a fluid waveform equivalent in amplitude and frequency to the sound pressure waves that initiated it. Adjacent to the oval window is another membranous surface called the round window, which allows movement of fluid through the cochlea by reacting in compliance to compression of the oval window. The oval window is covered by the stapes footplate, but the round window is

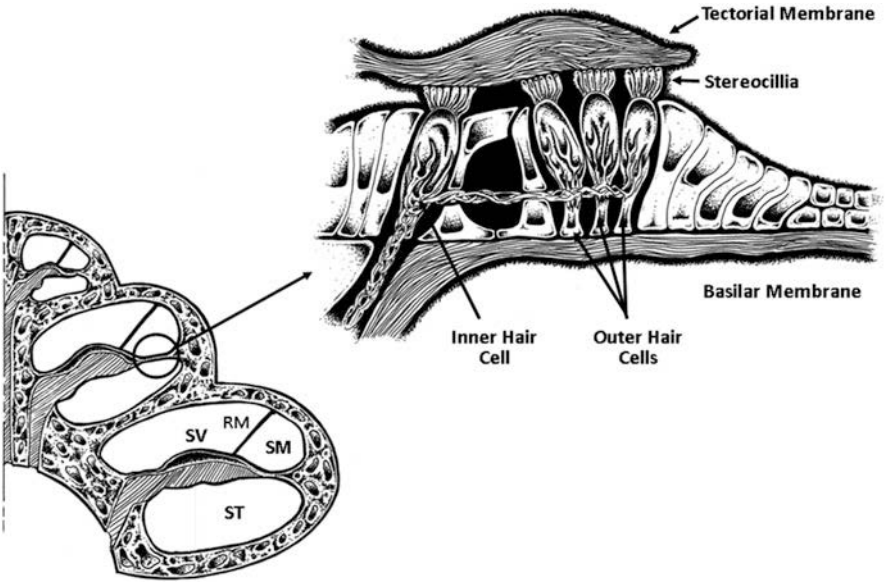

Fig. 2 Depiction of cochlear anatomy. *Left*: cross section of the cochlea. *Right*: expanded view of the organ of Corti. *SV* scala vestibuli, *SM* scala media, *ST* scala tympani, *RM* Reissner's membrane

exposed to the middle ear environment. This feature of the round window is important, as it is consequently permeable to large and small molecules, and therefore most drugs administered into the middle ear are absorbed into the cochlea at this site.

The cochlea is divided into three chambers by two other important membranes, the Reissner's membrane and the basilar membrane. These two membranes subdivide the semicircular canal into thirds, creating three distinct ducts or chambers: (1) the scala media (middle chamber), (2) the scala vestibuli (vestibular side chamber), and (3) the scala tympani (drum side chamber) (see Fig. 2). All three subchambers are filled with lymphatic fluid. The scala vestibuli and scala tympani contain a fluid called perilymph, while the scala media contains endolymph. It is important to note that the endolymph is the fluid environment surrounding the auditory hair cells. Perilymph and endolymph have unique ionic compositions suited to their functions in regulating electrochemical impulses of hair cells. The electric potential of endolymph is approximately 80–90 mV more positive than perilymph due to a higher concentration of potassium compared to sodium, a circumstance required for auditory hair cell depolarization.

As the pressure waveform conducted by the perilymph moves within the scala tympani, the basilar membrane also begins to move, which in turn causes movement of the tectorial membrane across the hair cells of the organ of Corti. Of critical importance is the functionality of the basilar membrane, as this specialized structure displays differential frequency conductivity as a function

of anatomic location. Lower frequencies cause the greater displacement of the membrane in the apex of the cochlea and the higher frequencies in the basal region of the cochlea.

The subsequent movement of the organ of Corti is also critical to auditory signaling because on the ventral side of the organ lie four rows of auditory hair cells. The first three rows of cells are referred to as outer hair cells, while the fourth is comprised of inner hair cells. The hair cells are distributed tonotopically throughout the length of the cochlea that is by selective frequency organization from base to apex. It is because of the specialized structure of the basilar membrane that transduction will only trigger neurotransmission within a circumscribed subpopulation of cells when stimulated by a specific and limited set of frequencies.

As denoted by the nomenclature, each hair cell possesses stereocilia which come into physical contact with the overlying stable tectorial membrane. Waveform movement of the basilar membrane and cell bodies of the hair cells elicits a shearing movement of the cells' stereocilia against the tectorial membrane. Many neurons in the CNS are noted for their ion-gated or voltage-gated channels. The stereocilia of the hair cells represent a mechanically gated ion channel. Normally, the shearing forces of a wavelike movement of the hair cells against a stable tectorial membrane bend the stereocilia which mechanically opens channels on the apical end of the hair cells and allows potassium to enter the cell triggering depolarization. This unique mechanically gated ion channel in the hair cells makes them uniquely sensitive to permeation by any novel or toxic small or large molecule that has migrated into the endolymph of the scala media.

Once depolarized, the outer and inner hair cells are responsible for different functions. The outer hair cells have specialized motors, which, when stimulated by the cell depolarization, change the shape of the cell. This action, called electromotility, allows for the outer hair cells to help the auditory system discriminate closely related frequencies.

In contrast to the outer hair cells, depolarization of the inner hair cells results in auditory neurotransmission. Following depolarization, voltage-gated calcium channels on the inner hair cell membrane open which signals trafficking of glutamate to the synaptic zone between the inner hair cell and afferent neurons. These neurons propagate signals up through the auditory pathway to the auditory cortex.

With knowledge of the complex interactions necessary for normal hearing, it can easily be understood how damage to, or interference with, any of the structures within the middle or inner ear can cause loss of auditory function. If loss of function is the result of drug exposure, it is important to be able to identify the underlying precipitating events initiating the toxic cascade and determine the long-term implications, including potential recovery, from the damage.

2 Ototoxicity Studies

Auditory safety studies are conducted under the guidance of the Food and Drug Administration's (FDA) draft document released in 2008 titled, "Nonclinical Safety Evaluation of Reformulated Drug Products and Products Intended for Administration by an Alternate Route." While guidance documents do not represent regulatory requirements per se, they do represent agency current thinking on critical elements of these study designs. If an NCE is expected to reach the middle or inner ear, the FDA's guidance document recommends that several endpoints should be evaluated by means of direct administration in order to adequately assess otological safety. The guidance document recommends the following endpoints: evaluation of actual exposure, evaluation of the auditory brainstem response (ABR) to stimulation under relevant toxic exposure conditions of the NCE, microscopic evaluation of relevant structural and cellular tissues, and a cellular mapping of damage (e.g., cytocochleogram analysis). Together, these recommended endpoints will help determine the toxicological impact of the administration of an NCE (e.g., drug, cosmetic, or environmental contaminant) prior to first human exposure.

2.1 Middle Ear Exposure

The first endpoint listed in the guidance document is the determination of middle and inner ear exposure. As in all other preclinical safety assessment protocols, it is important that dose selection is based on the pharmacokinetics of the NCE and that otic exposure is primarily regulated by the drug's ability to penetrate the tympanic membrane and/or the round window (administration and distribution). Perilymph collections can be a useful tool to determine otic exposure.

There are several variables to consider when evaluating perilymph drug concentrations:

1. The NCE may differentially distribute throughout the cochlea, which can make it difficult to achieve consistent concentrations if samples are not collected from the same region.
2. When large volumes of perilymph are removed from the cochlea for analysis, there is a potential for cerebrospinal fluid (CSF) replacement which may contaminate or dilute the actual drug concentration in a given sample.
3. The collection of multiple perilymph samples in a single animal requires the use of a microcatheter, which can be technically demanding and lead to issues with sample quality.

These variables can be minimized by design changes to the standard experimental protocol. It has been shown that utilization of smaller volumes, collected at least 4 mm from the round window, produces negligible CSF contamination. These small sample aliquots will yield more consistent drug concentrations regardless of differences in drug distribution (Salt et al. 2003). Additionally, although methods

exist to perform multiple perilymph collections from the same animal, most experiments utilize a one sample-one time point design (Bunting et al. 2004; Hoffer et al. 2011; Salt et al. 2003, 2006; Yang et al. 2008). This sampling strategy helps to reduce the technical demands of the procedure and provides better quality data to establish the pharmacokinetics of the drug in the inner ear.

2.2 Auditory Brainstem Response

The next endpoint listed in the FDA's guidance document is evaluation of the auditory brainstem response (ABR). In the clinic, audiologists are able to present sound stimuli with varying degrees of intensity to patients, and their behavioral or oral response to the processing of such stimuli will determine the individual's frequency thresholds across the test range imposed. In animal models, auditory thresholds cannot be based solely on behavioral responses, and therefore these thresholds are generally determined by conducting surface electrode evaluations of underlying neural processing. The ABR evaluation relies upon the interpretation of a series of multiphasic electroencephalograms (EEG) produced following a sound stimulus presented at descending intensities. When electrodes are placed on the surface of the scalp in a specific configuration, EEG waveforms induced by auditory signal presentations may be amplified and recorded. The resulting multicellular electrical responses become a visibly quantifiable representation of the neural processing of the applied auditory stimulus. The resultant waveform is thought to represent globally the functional propagation of neural activity throughout the auditory pathway. The ABR waveform consists of up to five distinct phasic components representing peak neural responses generated within different regions along the auditory pathway from the ear to the central thalamic information processing control sites: waves I and II from the auditory nerve, wave III from the cochlear nucleus, wave IV from the superior olivary complex, and wave V from the lateral lemniscus (Roeser et al. 2000) (see Fig. 3). Using the waveform as an indicator of audition, a stimulus may be presented at different sound pressure levels (decibels) and a minimal detectable level or threshold can be determined.

The ABR is a useful tool for testing functional hearing, as multiple frequencies may be assessed in one evaluation. Since the cochlea is tonotopically organized, such assessments help to determine functional loss at specific locations in the cochlea.

When hearing loss is present following NCE administration, one of three categories is typically observed: conductive, sensorineural, or mixed:

1. In sensorineural hearing loss, the primary cause of the hearing deficit is damage to the auditory hair cells (Abi-Hachem et al. 2010). Interestingly, most drug-induced auditory hair cell damage begins in the basal region of the cochlea, which will result in increased ABR thresholds at higher frequencies first and then affect lower frequencies as damage progresses overtime (Poirrier et al. 2010; Song et al. 1997).

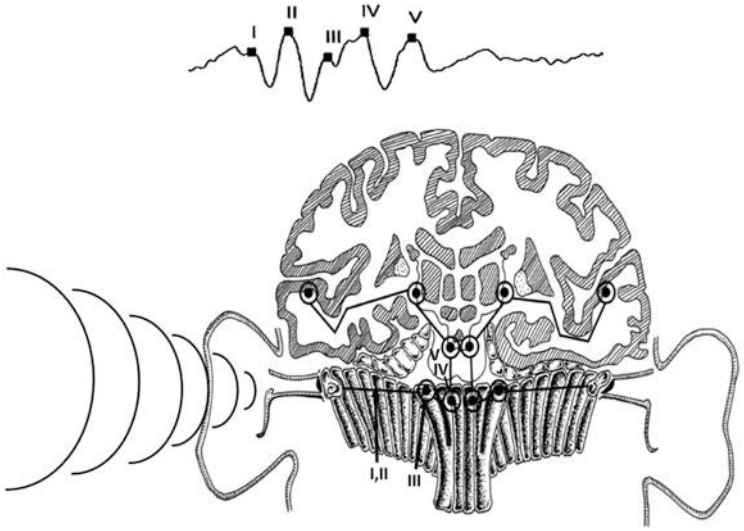

Fig. 3 Example ABR waveform with corresponding auditory pathway locations. Waves I and II represent activity in the auditory nerve, wave III represents activity in the cochlear nucleus, wave IV represents activity in the superior olivary complex, and wave V represents activity in the lateral lemniscus

2. Conductive hearing loss is caused by damage to, or interference with, the outer or middle ear structures associated with amplifying and conducting sound waves to the inner ear (e.g., bony structures of the malleus, incus, and stapes). Conductive hearing loss in the context of drug administration is usually a consequence of the route of administration and/or local effects induced within the middle ear. Conductive hearing loss can be detected in the form of increased lower-frequency ABR thresholds only (Roland 1997). Presence of fluid, local irritation or inflammation, and tympanic membrane perforations (via injection) are all variables associated with drug administration that can cause reversible conductive hearing loss (Roland 1997).
3. Mixed hearing loss is a blend of sensorineural and conductive hearing loss and generally results in increases in all ABR frequency thresholds.

2.3 Otic Microscopy

Since many systemically administered drugs will have the potential to interact with auditory structures and function, the FDA's guidance document also suggests administering a compound directly to the middle ear. Therefore it is essential to evaluate the middle ear with regard to structural and mucosal damage following

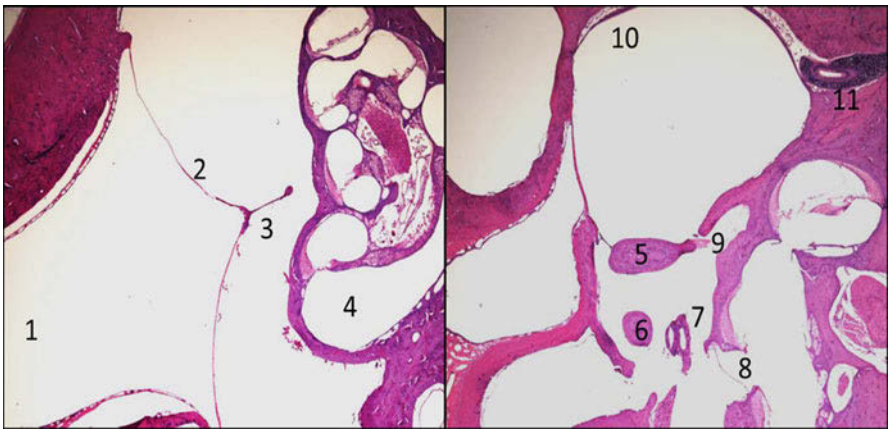

Fig. 4 Photomicrographs of hematoxylin and eosin-stained sections of the middle ear from a guinea pig. *1* external auditory canal, *2* tympanic membrane, *3* junction of the tympanic membrane and malleus, *4* cochlea, *5* malleus, *6* incus, *7* stapes, *8* round window, *9* tensor tympani, *10* middle ear mucosa, *11* entrance to the eustachian tube

direct intratympanic administrations. Additionally, the results of otic microscopy may be employed to support or refute any ABR findings suggestive of conductive hearing loss.

When approaching the evaluation of the middle ear, it is important to identify the structures intrinsic to sound conduction. If the temporal bone is oriented precisely, it is possible to obtain sections of the tympanic membrane, ossicular chain, tensor tympani, round window, cochlea, and middle ear mucosa (see Fig. 4). Evaluation of these structures will provide insight into how a drug is interacting with the middle ear environment. Signs of inflammation, fibrosis, and necrosis can result from direct drug administration into the middle ear. Investigations including these types of effects may help to better characterize conductive changes in hearing that are a result of drug administration.

2.4 Cytocochleograms

The cytocochleogram is a form of microscopy aimed at quantifying damage to the auditory hair cells throughout the cochlea. Specifically, cytocochleograms are a tonotopic representation of auditory hair cells as a function of distance from a fixed point in the cochlea. As indicated in the auditory physiology section, auditory hair cells are distributed throughout the cochlea and, based on their location, will only be stimulated by specific frequencies. Therefore, concomitant analysis of the cytocochleogram allows for definitive interpretation of ABR threshold data. Where otic microscopy allows for investigation into the potential cause(s) of

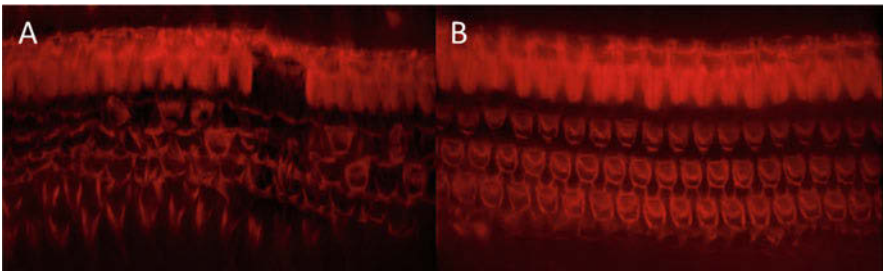

Fig. 5 Confocal images of fluorescently stained surface preparations of the basal region of the organ of Corti from a (**a**) drug-treated and (**b**) control animal

conductive hearing loss, inclusion of a cytocochleogram allows for a more detailed evaluation of the potential for sensorineural hearing loss.

There are several variations used for assessing hair cells, but the most common method employed utilizes fluorescent staining of surface preparations of the organ of Corti. Once highlighted with cellular stains, microscopy allows the researcher to physically count the hair cells that are present and missing (see Fig. 5). The percentage of missing hair cells throughout the cochlea can thus be calculated. This type of analysis allows an investigator to assess changes found in an ABR evaluation at specific frequency bands and correlate these to hair cell data from a corresponding region of the cochlea.

3 Species Selection in Ototoxicity Studies

Appropriate species selection for ototoxicity studies is essential for successful characterization of the ototoxicity of a compound. When selecting a model for ototoxicity studies, there are two important factors to consider. First and foremost is the bioavailability of the compound within the model selected. It is important to select a species in which there is adequate absorption though the round window and/or the tympanic membrane, if applicable. If perilymph concentrations of the NCE do not reach levels comparable to that of the human, the preclinical data serves no translational purpose.

The second factor to consider when choosing a research model is the feasibility of collecting the necessary data to fulfill study objectives. The size of the animal alone can cause problems with regard to the feasibility of ABR data collection and subsequent histopathology. The remaining portion of this section will discuss species-specific advantages and limitations as these are applied within the context of preclinical ototoxicity study design.

3.1 Mouse

The mouse is largely used in genetics-based research due to a large historical database of auditory parameters and well-defined genome allowing for extensive knockout work.

Positive Aspects
- Species is inexpensive allowing for the use of larger group sizes.
- Well-established methods and historical data for audiometry and histopathology, including cytocochleogram analysis.
- Relative size of the experimental subject allows for less required test material.
- CBA mouse strain presents with a relatively slow rate of development of age-related deafness and is appropriate for longer-term studies (Guimaraes et al. 2004; Li and Hultcrantz 1994; Spongr et al. 1997).

Negative Aspects
- Large variety of sub-strains presenting with varying degrees of age-related sensorineural deafness occurring early in life (Erway et al. 1993; Johnson et al. 2000; Ohlemiller 2006).
- Rapid advancement of age-related deafness may make control audiometry and hair cell data difficult to interpret and compare to drug-treated groups.
- May limit study duration due to increased auditory damage with age.
- Cochlear lesions are difficult to induce via drug administration and therefore may not be appropriate for some types of safety evaluations (He et al. 2009; Hirose and Sato 2011; Poirrier et al. 2010; Qureshy et al. 2011; Wu et al. 2001).

3.2 Rat

The rat is a common model used in toxicology research, but has also been shown to be a useful model for ototoxicity safety testing.

Positive Aspects
- Species is inexpensive allowing for the use of larger group sizes.
- Sprague-Dawley, Long-Evans, and Fischer rats can be used on longer-term studies as they generally present with age-related hearing loss after 12 months of age (Bielefeld et al. 2005; Buckiova et al. 2007; Keithley and Feldman 1982; Popelar et al. 2006; Syka 2010).
- Species is responsive to clinically ototoxic drugs making it an appropriate safety model (Lataye et al. 2003, 2004; Lee et al. 2011; Okur et al. 2007; Sullivan et al. 1988).

Negative Aspects
- Source for historical data is less developed than that of the mouse or guinea pig.
- On long-term studies (>6 months), species can grow as large as 1 kg which may complicate terminal auditory assessments and increase the time necessary to perform histopathology due to augmented bone density.

3.3 Guinea Pig

The albino and pigmented guinea pigs are commonly used in preclinical and academic research. The morphology of the middle ear of a guinea pig is proportionally and structurally similar to that of humans making it an excellent safety model.

Positive Aspects
- Species is inexpensive allowing for the use of larger group sizes.
- Well-established methods and historical data for audiometry and histopathology, including cytocochleogram analysis.
- Species may be used on longer-term studies as age-related hearing loss has been reported at 12 months of age and older (Coleman 1976; Nozawa et al. 1997; Proctor et al. 1998).
- Species is responsive to clinically ototoxic drugs making it an appropriate safety model (Aquino et al. 2008; Kawamoto et al. 2003; Lee et al. 2011; Matsuda et al. 2000; Nakagawa et al. 1998; Pawlowski et al. 2010).
- Species is commonly used for middle ear catheterization and/or direct middle ear administration of drugs (Agterberg et al. 2008; Lemke et al. 2009; Lin et al. 2008; Takumida et al. 1999; Tom 2000; Wang et al. 2003; Zappia and Altschuler 1989).

Negative Aspects
- On long-term studies (>6 months), species can grow as large as 1 kg which may complicate terminal auditory assessments and increase the time necessary to perform histopathology due to augmented bone density.

3.4 Chinchilla

Chinchillas have historically been used in topical auditory safety evaluations due to their large external auditory canal (Daniel et al. 2007, 2012; Peleva et al. 2011).

Positive Aspects
- Species is inexpensive allowing for the use of larger group sizes.
- Well-established methods and historical data for audiometry and histopathology, including cytocochleogram analysis.

- Species may be used on longer-term studies as age-related hearing loss has been reported at 3 years of age and older (McFadden et al. 1997).
- Species is responsive to clinically ototoxic drugs making it an appropriate safety model (Henderson et al. 2000; Hu et al. 1999; McFadden et al. 2002; Whitworth et al. 2004).
- Transtympanic administration is a common route in this species (Daniel and Munguia 2008; Munguia et al. 2010; Nader et al. 2007).

Negative Aspects
- Animal availability can be limited due to a small number of licensed distributors.

3.5 Large Animals

Large animals used for ototoxicity safety assessments are less common than those species listed above. Large animals would only be used for safety assessments if bioavailability is insufficient in those species listed above, the specific indication is for large animal veterinary use, and/or a study design requires a unique endpoint, i.e., large perilymph sampling volume. Common large animal species used in ototoxicity safety testing are cats (Igarashi and Oka 1988; Lenarz et al. 2007; Webster et al. 1971) monkeys (Carey et al. 2005; Hawkins et al. 1987; Yokota et al. 1984), and dogs (Kelley et al. 2010; Mills et al. 2005; Sockalingam et al. 2002).

Positive Aspects
- Species may allow for better bioavailability or cochlear drug distribution.
- Large middle and inner ear allows for better access for surgical manipulations.

Negative Aspects
- Species is more expensive to purchase and maintain, which may be a limitation for group size.
- Sources for historical data are limited.
- Temporal bone density may require extended decalcification time to obtain quality histopathology data.
- Increased animal size may require the use of more drug product (milligram/kilogram basis) compared to small animal models.

4 Designing an Ototoxicity Study

The design of an ototoxicity study is driven by the route and type of compound being evaluated. Within the FDA's guidance document, it is suggested that the drug be administered directly into the middle ear and also that the drug's safety be assessed on short- and long-term studies. Therefore to attain drug exposure, it is

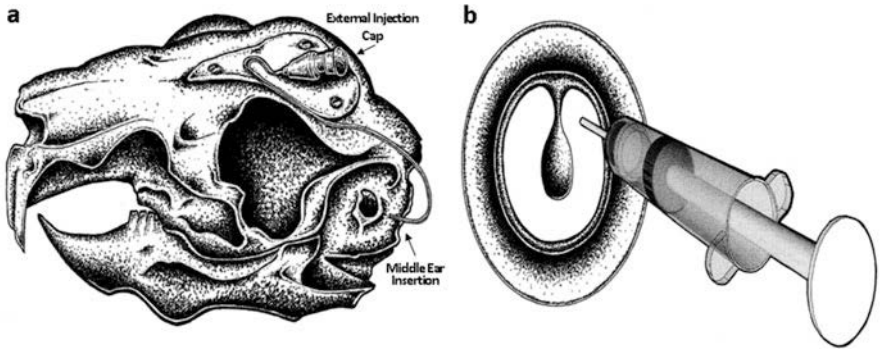

Fig. 6 Illustrations of (**a**) middle ear catheterization and (**b**) transtympanic drug administration

necessary to determine the compound's capacity for entering the middle ear through the tympanum. If a compound has poor absorption through the tympanum, there are two alternative methods for direct drug administration.

The first method is to instrument the animals with a middle ear catheter which allows for direct administration of serial treatments. For this procedure, animals are anesthetized, and a catheter is placed into the middle ear with the opposing end attached to an external injection cap secured to the external cranium (see Fig. 6a). Following instrumentation, animals generally recover without any hearing deficits or health effects.

The second method for direct drug administration to the middle ear involves transtympanic injections. The tympanum readily heals following perforation, rendering the transtympanic injection a viable alternative. A transtympanic injection requires the animal to be anesthetized, with subsequent insertion of a needle through the tympanum. Generally, the drug is injected into the middle ear in the area around the round window niche. The drawback to this method is that perforating the tympanum can result in temporary auditory threshold shifts of 20 dB or larger at lower frequencies depending on the perforation size (Mehta et al. 2006). Allowing a recovery or incorporating additional ABR time points following dosing will prevent reporting false threshold increases.

Selection of the number of animals to be placed on each study should also be strongly considered with regard to variability in ABR and cytocochleogram data. Generally, group sizes range from 5 to 8 animals, but may vary in consideration of effects induced by the drug treatment (Conlon et al. 1999; He et al. 2009; Hill et al. 2008; Himeno et al. 2002; Matsuda et al. 2000; Sha and Schacht 2000; Yumusakhuylu et al. 2012). Group sizes should also be determined based on the ability to obtain the necessary endpoints from one animal, which will be discussed later in this section.

The next step in designing an ototoxicity study is to determine the number of necessary functional hearing endpoints. Most studies begin by performing pre-study hearing tests on all animals to identify those animals with normal hearing

thresholds. Several factors should be considered prior to the initiation of auditory prescreening. The first factor to consider is that if a study requires middle ear instrumentation, hearing should be assessed both before and after surgery or alternatively at least following surgery to ensure normal hearing before drug administration. The duration of the study and number of doses will also dictate how additional ABR time points are integrated into the study design. At minimum, an ABR should be conducted at the termination of the study to determine if any hearing deficits were induced by administration of the drug. ABR time points may also be integrated after dosing to show possible recovery of hearing caused by a local inflammatory response.

The definitive data in an ototoxicity study is generated by the otic microscopy and cytocochleogram analysis. An inherent obstacle associated with integrating otic histopathology into your study is that one ear cannot be used to generate both histopathological and cytocochleogram data. The methods required to harvest the organ of Corti for cytocochleogram analysis involve removing most of the middle ear structures. There are two possible ways to approach designing a study successfully incorporating these endpoints while still maintaining statistical power.

The first approach can be employed when bilateral administration is feasible. In this case, each middle ear in an animal is treated, and therefore one ear may be used for histopathology and the other for cytocochleogram analysis. This approach will reduce the number of animals on study and may allow for better correlation of middle ear histopathological and inner ear cytocochleogram analysis by reducing the inter-animal differences in drug distribution and metabolism.

The second approach is utilized when a unilateral drug administration is employed. In the case where only one middle ear is treated in an animal, it is important to understand that the initial group number will be have to be doubled to generate each type of data.

5 Supplemental Endpoints

There are many additional ways to assess ototoxicity that are not required endpoints by the Food and Drug Administration, but could provide additional insight into the interactions a compound may have with those structures associated with hearing. This section will highlight some potential evaluations which could be integrated into a safety study without affecting the overall design.

5.1 Distortion Product Otoacoustic Emissions

Distortion product otoacoustic emissions (DPOAEs) can be used to evaluate the function of the outer hair cells alone. DPOAEs are created as a by-product of outer hair cell motility following a specific stimulus presentation. When the basilar membrane moves in response to a sound, outer hair cells are stimulated and by electromotility aid in tuning specific frequencies to stimulate the inner hair cells

resulting in neurotransmission. The distortion product is produced when two stimuli are presented at similar frequencies, f2 and f1, one slightly more intense than the other. The two waves are thought to interact and create a sound wave that is of the frequency 2f1 − f2, which travels toward the input direction (Dalhoff et al. 2007). The resultant wave travels through the cochlea, out of the oval window, and through the middle ear where it is emitted from the tympanic membrane as a noise (Hall 2000). The noise can be averaged and the intensity defined for pretest and postdose evaluations.

The DPOAE evaluation can be conducted similarly to that of the ABR which allows it to easily be integrated into a study design without the need for additional animals. If used correctly, DPOAEs can provide direct information regarding outer hair cell viability when increases in ABR thresholds are observed. The DPOAE is a real-time tool for understanding how a drug affects outer hair cells without the need for animal sacrifice and subsequent hair cell analysis.

5.2 Spiral Ganglion Evaluations

The spiral ganglia are the first bundle of neurons projecting from the hair cells which lead to the auditory nerve. These neurons are located in the cochlea and can be viewed in midmodiolar sections (middle plane) of the cochlea. As described in the next section on ototoxic drugs, some compounds can directly affect the spiral ganglia resulting in ultrastructural damage and/or cell death. By using serial or step sections through the cochlea, spiral ganglion measurements can be collected from different regions of the cochlea. Together with hair cell data, these measurements can be used to determine if the changes are a direct result of drug-induced damage or a neurodegenerative effect from lack of stimulation due to hair cell loss. Spiral ganglion assessments can easily be inserted into a standard study by expanding the cochlear sectioning method conducted during microtomy. The most important aspect when taking cochlear sections is to ensure that the same sampling and orientation procedure is followed in each ear so that the data are comparable across study groups.

5.3 Semiquantitative Hair Cell Assessments

The main drawback to surface mount hair cell analysis is that it requires that the middle ear be dissected without analysis, thus yielding only an understanding of hair cell viability without any other information on damage that may be present in the remaining structures of the inner and middle ear. Although fluorescently stained hair cells allow for easy evaluation of hair cell integrity, simple hematoxylin and eosin stains may also enable hair cell assessment with light microscopy. Again using serial or step sectioning through the midmodiolar plane of the cochlea, hair cells may be evaluated in various regions which can be used to establish a cause for ABR threshold changes. Hair cell loss detected in the basal region of the cochlea

would elucidate any high-frequency hearing loss detected during an ABR evaluation. Similar to that of the spiral ganglia assessments, a hair cell evaluation can be integrated into a standard design by including an expanded cochlear sectioning method. Although only a small percentage of the total hair cells are amenable to analysis by this method, this technique allows for analysis of the middle and inner ear as a whole, providing a more connected data set.

6 Classes of Compounds Associated with Ototoxicity

There are multiple classes of compounds known to cause ototoxicity. Interestingly, some of these compounds are used clinically every day, but limited to those lower dosages not associated with ototoxic effects. The remaining text of this chapter will discuss the major classes of ototoxic compounds and will describe the proposed mechanism of action associated with causing ototoxicity.

6.1 Salicylates

Salicylates have been used for over a century for the treatment of pain and as a fever reducer. Salicylates reduce pain and fever by inhibiting cyclooxygenase, an enzyme responsible for producing prostaglandins which mediate inflammation and thermoregulation (Vane and Botting 1998). Over this century, there have been numerous reports showing salicylate-induced tinnitus and reversible sensorineural hearing loss (Day et al. 1989; Guitton et al. 2003; Halla and Hardin 1988; Halla et al. 1991). Researchers have shown with audiometric and histopathologic data that the primary target of the drug is the outer hair cells and their stereocilia (Pienkowski and Ulfendahl 2011). The hair cells themselves do not seem to undergo cell death, but their electromotility is impaired, which is thought to cause auditory threshold increases (Shehata et al. 1991; de Almeida-Silva et al. 2011; Yu et al. 2008). The effect is temporary, and following cessation of treatment, restoration of hearing begins within 24–48 h (Lue and Brownell 1999).

Another interesting aspect of salicylates is that chronic administration has been shown to cause neural degeneration of the spiral ganglion (Wei et al. 2010). Preclinical data suggest that death of the ganglion is a result of caspase-driven apoptosis caused by cyclooxygenase inhibition (Feng et al. 2010, 2011; Jung et al. 1997).

6.2 Loop Diuretics

Loop diuretics are compounds used to treat congestive heart failure, pulmonary disorders, renal failure, and hypertension (Roland and Rutka 2004). Loop diuretics increase water excretion by inhibiting NaCl reabsorption within the loop of Henle

in the kidney (Mozayani and Raymon 2004). Common loop diuretics possessing ototoxic properties at high doses are furosemide, bumetanide, and ethacrynic acid.

Data suggests that loop diuretics cause toxicity within the stria vascularis, the structure which provides blood flow to the cochlea (Forge and Brown 1982; Rybak et al. 1991). The stria vascularis contains Na-K-ATPases which maintain the high K content of the endolymph and the positive endocochlear potential in the scala media (Ikeda and Morizono 1989; Ikeda et al. 1997). It has been proposed that the damage to the stria vascularis causes a reduction in the endocochlear potential which then results in hair cell dysfunction (Comis et al. 1990).

6.3 Aminoglycoside Antibiotics

Aminoglycoside antibiotics are compounds used to treat gram-negative infections. This class of antibiotic acts on the 30S subunit of the bacterial ribosome causing DNA misreads and disruptions in protein synthesis, all resulting in cell death (Rybak and Ramkumar 2007). Common aminoglycoside antibiotics are gentamicin, neomycin, kanamycin, and streptomycin. With multiple classes of antibiotics on the market, aminoglycoside use has diminished due to dose-limiting nephrotoxicity and ototoxicity. However in recent years, aminoglycoside use has increased internationally due to a surge of multiresistant strains of tuberculosis (Vasconcelos et al. 2012).

In terms of ototoxicity, aminoglycosides affect hearing by causing damage to the auditory hair cells (Xie et al. 2011). Much research has been conducted on aminoglycosides in an effort to determine the mechanism of toxicity causing hair cell death. The primary mechanism related to hair cell death following aminoglycoside administration is attributed to free radical production (Sinswat et al. 2000; Song and Schacht 1996; Song et al. 1997; Takumida et al. 1999). The effects of aminoglycoside ototoxicity begin in the basal portion of the cochlea and then progress apically overtime. Interestingly, the graded difference in aminoglycoside-associated hair cell loss between the apex and basal regions of the cochlea has been attributed to the higher levels of glutathione (endogenous antioxidant) in the apical hair cells (Sha and Schacht 2000). It is thought that increases in cellular free radicals lead to a cascade of downstream events triggering apoptosis (Dinh and Van De Water 2009).

6.4 Platinum-Based Chemotherapeutics

Platinum-based chemotherapeutics are used clinically to treat soft tissue tumors. These types of platinum-based compounds bind to the nucleotide bases of DNA forming a bond which terminates cell division and triggers cell death (Kelland and Ferrell 2000). Although these compounds are highly effective anticancer therapies, their use commonly results to hearing loss. Common platinum-based chemotherapeutics are cisplatin, carboplatin, and oxaliplatin.

These compounds produce several toxic effects in the inner ear which have been correlated to hearing loss. Similar to the mechanism apparent with aminoglycosides, it appears these compounds produce free radicals which induce apoptosis in the auditory outer hair cells (Choe et al. 2004; Choi et al. 2013; Qu et al. 2012; Yumusakhuylu et al. 2012). These compounds also produce auditory neural damage by causing detachment of the myelin sheath surrounding the spiral ganglia, which is also thought to be a result of damage from free radicals (Lee et al. 2004; van Ruijven et al. 2005). Additionally, these drugs induce degeneration of the spiral ligament and stria vascularis (Rybak et al. 2007). Damage to each of these structures of the inner ear can produce effects resulting in deficits in auditory function.

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Gastrointestinal Safety Pharmacology in Drug Discovery and Development

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Abstract

Although the basic structure of the gastrointestinal tract (GIT) is similar across species, there are significant differences in the anatomy, physiology, and biochemistry between humans and laboratory animals, which should be taken into account when conducting a gastrointestinal (GI) assessment. Historically, the percentage of cases of drug attrition associated with GI-related adverse effects is small; however, this incidence has increased over the last few years. Drug-related GI effects are very diverse, usually functional in nature, and not limited to a single pharmacological class. The most common GI signs are nausea and vomiting, diarrhea, constipation, and gastric ulceration. Despite being generally not life-threatening, they can greatly affect patient compliance and quality of life. There is therefore a real need for improved and/or more extensive GI screening of candidate drugs in preclinical development, which may help to better predict clinical effects. Models to identify drug effects on GI function cover GI motility, nausea and emesis liability, secretory function (mainly gastric secretion), and absorption aspects. Both in vitro and in vivo assessments are described in this chapter. Drug-induced effects on GI function can be assessed in stand-alone safety pharmacology studies or as endpoints integrated into toxicology studies. In silico approaches are also being developed, such as the gut-on-a-chip model, but await further optimization and validation before routine use in drug development. GI injuries are still in their infancy with regard to biomarkers, probably due to their greater diversity. Nevertheless, several potential blood, stool, and breath biomarkers have been investigated. However, additional validation studies are necessary to assess the relevance of these biomarkers and their predictive value for GI injuries.

Keywords

Absorption • Biomarkers • Emesis • Gastric secretion • Gastrointestinal • Motility • Nausea • Safety pharmacology

List of Abbreviations

ADR	Adverse drug reaction
AE	Adverse effects
ANS	Autonomic nervous system
ATP	Adenosine triphosphate
CCK	Cholecystokinin
CFD	Computational fluid dynamic
CNS	Central nervous system
CRP	C-reactive protein
DAO	Diamine oxidase
DDI	Drug–drug interaction
DRF	Dose-range finding

ECL	Enterochromaffin-like cells
EFS	Electrical field stimulation
EMA	European Medicines Agency
ENS	Enteric nervous system
FDA	Food and Drug Administration
FTIM	First time in man
GE	Gastric emptying
GI	Gastrointestinal
GIT	Gastrointestinal tract
GLP	Good laboratory practice
ICC	Interstitial cells of Cajal
ICH	International Conference on Harmonisation
MMC	Migrating myoelectric/motor complex
MTD	Maximum tolerated dose
MW	Molecular weight
NCE	New chemical entity
NK	Neurokinin
NSAID	Nonsteroidal anti-inflammatory drug
OECD	Organisation for Economic Co-operation and Development
PACAP	Pituitary adenylate cyclase-activating enzyme
PAK4	p21-activated kinase
PMDA	Pharmaceuticals and Medical Devices Agency
SBT	Sucrose breathing test
SITT	Small intestine transit time
SP	Safety pharmacology
SS	Somatostatin
SSRI	Selective serotonin reuptake inhibitor
TCA	Tricyclic antidepressant
VIP	Vasoactive intestinal peptide

1 Introduction: Impact of Drug-Induced Gastrointestinal Injury on Drug Development

Over the last few decades, GI effects preclinically do not historically contribute highly to halted drug development, with fewer than ~2–3 % compounds discontinued from development or withdrawn from the market due to GI safety issues (Redfern et al. 2010). However, the remit of safety assessment testing is being reshaped to a certain extent by patient safety and clinicians who are mindful of the impact GI effects can have in clinical trials and post-marketing.

A recent analysis looking at the impact of adverse effects (AEs) and adverse drug reactions (ADRs) by organ function and throughout the pharmaceutical life cycle highlights the contribution of the various target organ systems to drug

attrition, incidence of serious ADRs, label ADRs, and involvement in withdrawal from sale (Redfern et al. 2010). The analysis concludes that around 14–23 % of drugs are associated with GI AEs or ADRs during clinical development (Redfern et al. 2010). The analysis also shows the marked impact that GI effects have in the clinic with a high incidence of GI-related ADRs in Phase I (23 %) and Phase III (67 %), figures which are higher than for the cardiovascular system and on a par with nervous system effects (Redfern et al. 2010). In an updated analysis concentrating on 2010 data only, GI effects were shown to display an increased contribution to clinical attrition (increase from 5 % to 11 %), while prescribing restrictions, ADRs on label and withdrawal from sales have decreased (Redfern et al. 2011).

GI AEs are generally not life-threatening, but the presence of recurring GI AEs and ADRs has the potential to greatly reduce the quality of life for patients, possibly resulting in reduced drug compliance and a further impairment of already fragile physical and mental states. Data from various sources shows that GI AEs are common; 18 % of total ADRs are reported to be associated with the GI system (Lewis 1986), and it has been reported that 20 % of Phase I clinical trial subjects reporting serious ADRs were reporting GI ADRs and that these ADRs often led to hospitalization (20–40 % patients) (Lewis 1986).

The GI AEs most likely to affect quality of life such as diarrhea are common, and a large number of drugs are associated with this particular side effect (>700) (Chassany et al. 2000). While most GI AEs are not life-threatening, in rare instances, severe GI events can develop. For example, the onset of complicated upper and lower GI tract complications, gastroduodenal ulcers, GI bleeding, and/or perforation is associated with the long-term use of nonsteroidal anti-inflammatory drugs (NSAIDs). NSAID use in the United States is estimated to be responsible for >100,000 hospitalizations and 17,000 deaths per year (Whittle 2003), while a prospective analysis of ~19,000 United Kingdom patients reported 17 deaths attributed to GI ADRs, most of which were attributed to NSAID use (Pirmohamed et al. 2004). Mortality can therefore be an issue, albeit a rare one.

Taking all of this information into account, there is clearly a need for improved and/or more extensive GI screening of candidate drugs in preclinical development, which may help to better predict clinical effects.

The term GI adverse effect encompasses a large number of signs and symptoms. GI AEs and ADRs can affect the GI tract in different ways, giving rise to a wide range of effects including nausea and vomiting, ulceration, inflammation of the intestine, altered fecal output, and abdominal pain/discomfort. The majority of GI AEs observed in the clinic are functional in nature, with fewer of pathological causation. They include altered GI transit, which can manifest itself as diarrhea and/or constipation (Ghahremani 1999; Gore et al. 1999; Lat et al. 2010). The fact that most GI AEs reported in the clinic are functional in nature should be driving a move toward establishing and incorporating many more GI-specific functional endpoints into safety pharmacology (SP) testing.

The presence of GI AEs is not restricted to a particular drug class (Table 1); antipsychotics, cytotoxics, antidepressants, antimicrobials, and anti-inflammatories

Table 1 Some drug classes that have gastrointestinal side effects in clinical use

Therapeutic class	Most notable examples	Side effect
Analgesics/anti-inflammatories	NSAIDs	Gastroduodenal ulcers
Antidepressants	TCAs, SSRIs[a]	Nausea, diarrhea, constipation
Antipsychotics	Chlorpromazine	Xerostomia (dry mouth)
Anticoagulants	Warfarin	Upper GI bleeding
Anticholinergics, opiates, sympathomimetics, antihistamines	Muscarinic antagonists[b]	Dry mouth, constipation
Antimicrobials	Penicillins, erythromycin, tetracyclines	Diarrhea, esophageal inflammation
Cytotoxic	5-fluoro-2′-deoxyuridine	Perforated ulcers, GI bleeding

NSAIDs nonsteroidal anti-inflammatory drugs, *SSRI* selective serotonin reuptake inhibitors, *TCA* tricyclic antidepressants
[a]Wang et al. (2014)
[b]Scully (2003)

have all been shown to result in GI AEs in the clinic (Valentin et al. 2011). From Drug Information Association Daily Alert data, within the 11 % of delayed or halted compounds, therapy areas included type II diabetes, anti-inflammatory conditions, cancer, depression, infection, and acne (Valentin et al. 2011). GI AEs are not only associated with varied drug classes but also a wide range of therapy areas and chemical classes; for example, oncology compounds have previously been shown to have a larger incidence of GI AEs and some, for example, NSAIDs, have more serious ADRs—gastroduodenal ulcers, GI bleeding, and/or perforation (Lanas and Ferrandez 2006; Lanas and Hunt 2006; Leong and Chan 2006).

In addition to the detrimental impact GI AEs and ADRs can have in the clinic, altered GI function can also result in dose-limiting toxicity and cause altered pharmacokinetic profiles in preclinical studies. Issues associated with GI AEs occur in all areas of drug development. The customer base for safety pharmacologists is large, and therefore we should be spending more time trying to increase awareness of gaps in preclinical assessment of GI liability.

2 Anatomy and Physiology

The digestive tract is a continuous tube that extends from the mouth to the anus. It is divided into upper and lower tracts. The upper tract contains the pharynx, esophagus, stomach, and duodenum up to the major papilla. The lower tract contains the duodenum from the major papilla, jejuno-ileum, colon, cecum, and rectum. Associated digestive organs are the teeth, tongue, salivary glands, liver, gall bladder, and pancreas. The GI tract is involved in the ingestion, digestion, absorption, and excretion of food as well as xenobiotics. While the basic structure of the

GIT exhibit considerable similarities across species, there are significant differences in the anatomy, physiology, and biochemistry between human and commonly used laboratory animals (detailed review can be found in Kararli 1995).

2.1 Gastrointestinal Gross Structure

The GI organs are covered with the peritoneum forming the outer serous membrane. The peritoneum binds organs to one another and suspends the bowel in the abdominal and pelvic cavity. The length of the GIT in adult human is about 9 m, about 5 m in dog, and about 1.5 m in rat. The wall of the stomach and small intestine consists of three muscle layers (Fig. 1): outer longitudinal, two middle circular muscle, and inner muscularis mucosa located between submucosa and mucosa. In human colon, the longitudinal muscle fibers are arranged in a band like taenia coli which gives the sacculations or haustra appearance of the different sections of the colon. The rat and dog colon is not sacculated. In humans and dogs, the cecal region is very small, while guinea pigs and rats have large cecum. These species differences should be taken into consideration and may affect the selection of laboratory animals for specific endpoints and hence the translation of animal data to human.

The luminal surface of the GIT is covered by mucosal cells that produce mucus to protect the mucosa from acids and harmful luminal conditions and secretes digestive enzymes and regulatory peptides specific to each section of the GIT (Hall 2011). Half of the GIT fluid is secreted by the salivary glands, pancreas, and liver. The stomach mucosa contains different types of cells with distinct

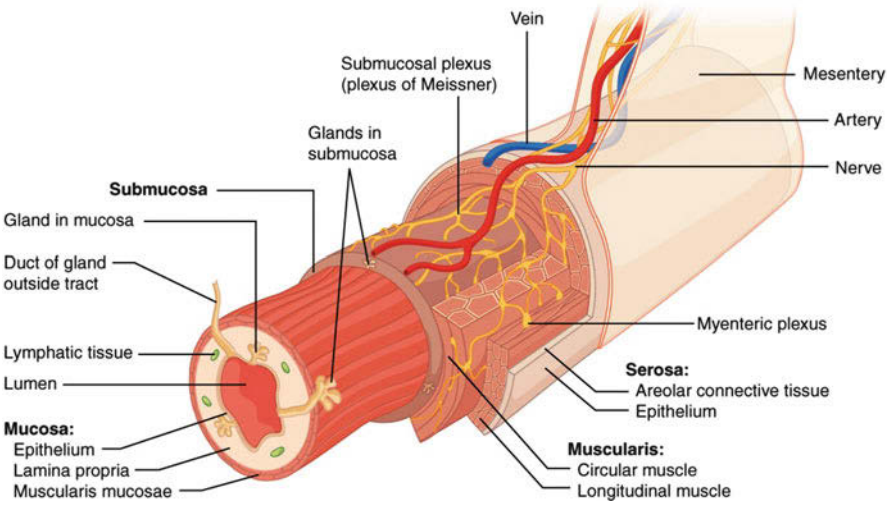

Fig. 1 Layers of the gastrointestinal tract (*Source*: OpenStax College, Rice University, USA (http://commons.wikimedia.org/wiki/File:Layers_of_the_GI_Tract_english.svg))

distribution that secretes H^+ (parietal cells), pepsin (chief cells), histamine (enterochromaffin-like cells, ECL), and GI hormones like gastrin, somatostatin (SS), and ghrelin (enteroendocrine cells). ECL and SS cells play an important role in the stimulation and inhibition of acid secretion, respectively. The duodenum contains Brunner's gland that secretes alkaline to neutralize the acidic gastric chyme. The absorptive and secretory cells of the small intestine mucosa secrete about 1.8 L/day of water, electrolytes, and digestive enzymes. Enteroendocrine cells which produce and release a variety of hormones are present along the mucosa of the small intestine with a distribution related to its particular action in the digestive process. Secretin and cholecystokinin (CCK) are released from endocrine cells in the duodenum to regulate pancreatic secretion and gall bladder emptying. Motilin, ghrelin, glucagon-like peptide-1, pancreatic polypeptide, and peptide YY are among other GI hormones that have been suggested to be involved in the regulation of GI motility and energy homeostasis (Hellström et al. 2006; Chaudhri et al. 2006; Murphy and Bloom 2006; Rehfeld 2014). About 90 % of the total body serotonin is located in the enterochromaffin cells.

The intrinsic innervation of the GIT is considered as a separate autonomic division, called the enteric nervous system (ENS) (Schemann and Neunlist 2004). Neurons and enteric glia of the ENS are embedded within the gut wall and arranged in three plexuses (Hall 2011). The myenteric (Auerbach's) plexus is located between the inner and outer circular muscle layers and the submucosal (Meissner's) plexus between the circular muscle and muscularis mucosa. The deep muscle plexus separates inner and outer circular muscle layers in the small intestine. In the large intestine, it is located at the inner border of circular muscle and is called submucosal plexus.

Neurons of ENS can be classified by their main function into sensory neurons, activated by thermic, chemical, or mechanical stimuli, and interneurons, transmitting signals between sensory and motor neurons (Wood 2008). Excitatory neurons project in the oral direction are cholinergic (muscarinic M_3 receptors), peptidergic (neurokinin 1, 2, 3 receptors), and serotonergic (5-$HT_{3,\ 4}$ receptors). Aborally projecting inhibitory neurons mediate inhibition through nitric oxide, vasoactive intestinal peptide (VIP) and ATP, and pituitary adenylate cyclase-activating enzyme (PACAP).

2.1.1 Gut–Brain Axis

The combined functioning of gastrointestinal motor, sensory, and central nervous system (CNS) activity is termed the brain–gut axis. The gut–brain axis comprises a network of neurons belonging to the ENS which is contained in the wall of the GIT and to the autonomic nervous system (ANS) that connect to the central nervous system by neurons traveling through the vagus, splanchnic, mesenteric, and pelvic spinal nerves to innervate the abdominal viscera (Furness 2006; Udit and Gautron 2013). The gut–brain axis is important in the regulation of food intake (Bewick 2012).

Though ENS can work independently of the CNS and can accommodate local secretory and motility activities, its function is modulated through extrinsic nerves

from the CNS via the ANS (Goyal and Hirano 1996; Furness 2006). There are large numbers of neurotransmitters localized in the ENS, and nearly every mediator found in the CNS is found in the ENS, which has even been described as a "second brain" or "enteric brain" (Gershon 1999). Therefore, even the development of highly selective compounds will possess some off-target interaction in the GIT.

2.2 Physiology

Ingested food and xenobiotics are subjected to multiple processes during their passage through the GIT. These processes include mucosal secretion, mixing, and propulsion by smooth muscle contraction, mechanical and chemical breakdown to small molecules, absorption by the mucosa, and passage of nutrients or xenobiotics to the blood and lymph vessels. Indigestible substances and metabolized xenobiotics excreted through the bile are eliminated by defecation.

Following ingestion, material (food or xenobiotics) is subjected to fragmentation by the teeth and humidification and predigestion by saliva in the oral cavity (site of the opening of the salivary gland ducts). The stomach serves as a reservoir of ingested food and performs mechanical and chemical breakdown of food leading to formation of a semifluid material called chyme and the emptying of chyme into the duodenum at a controlled rate. The presence of chyme in the small intestine stimulates local and peripheral nervous signals and hormonal factors which regulate intestinal motility and secretion. Emptying of chyme from the ileum into the large intestine is controlled by the ileocecal sphincter. In the proximal part of the colon, the absorption of water and electrolytes from chyme takes place to form solid feces that are stored in the distal part of the large intestine until fecal material is forced into the rectum by intense mass movements. Defecation reflexes involve rectal ENS and sacral division of the parasympathetic nervous system.

To identify drug-related effects on GI functions, researcher should take into consideration that a potential off-target drug effect might be on the GI mucosa, smooth muscle, or regulatory mechanisms of the GI function at the level of ANS, ENS, and GI hormones.

2.2.1 Gastrointestinal Motility and Transit

The ingested food is first chewed and mixed with salivary secretions in the mouth. After being swallowed, it progresses along the esophagus to reach the cardia. Contractions of the musculature along the bowel are organized to achieve mixing of chyme with biliary and pancreatic secretions, circulation of the contents so that they come into contact with mucosal cells, and transport of the contents in a net aboral direction. The propulsion of contents along the GIT varies according to the specific motor activity at different parts of the gut. The smooth muscle of the gastrointestinal tract is excited by almost continual slow, intrinsic electrical activity along the membranes of the muscle fibers. This activity has two basic types of electrical waves: (1) slow waves and (2) spike potentials, the counter part of muscle contraction.

GI smooth muscle fibers are electrically connected with each other through large numbers of gap junctions, allowing the smooth muscle fibers to work as a syncytium with good electrical coupling (Daniel et al. 2001). The spontaneous myogenic activity and many rhythmic motor activities of the GIT are generated and propagated by specialized pacemaker cells called interstitial cells of Cajal (ICC) (Huzinga and Chen 2014). ICC are located in the myenteric plexus and submucosal plexus of the small and large intestine, respectively. ICC have distinct regional distribution patterns, are interconnected by gap junctions, and transmit their pacing current to the smooth muscle by a synaptic-like contact (Komuro 2006). Recent emerging evidence suggests a relationship between ICC injury and GI motility disorders and diseases like diabetic gastroenteropathy, ulcerative colitis, and Crohn's disease (Huzinga and Chen 2014).

The GI contractions in humans, dogs, and rats are organized into fasted and fed motility patterns in relation to the digestive state of the bowel (Szurszewski 1969; Ruckebusch and Fioramonti 1975; Vantrappen et al. 1977). Fasted or interdigestive pattern, the so-called migrating myoelectric (motor) complex (MMC), is characterized by cyclic occurrence of an intense burst of regular spiking activity (phase III) that propagates from the stomach to the ileum proceeded by a longer period of slow waves or quiescence (phase I) and a period of randomly occurring spiking potentials or contractions (phase II). The MMC length varies between different species; in fasting humans, it recurs approximately every 90–120 min (Deloose et al. 2012), in dogs about 100 min (Szurszewski 1969), and in rats about 15 min (Ruckebusch and Fioramonti 1975). In humans, dogs, and rats, feeding causes immediate disappearance of the MMC and initiates a "fed" motility pattern consisting of short bursts of contractions (segmentation contraction) without distinct phases as MMC. The physiological function of MMC is not completely understood, but has been suggested to function as a "house keeper" to clean the small intestine in-between meals from digestive derbies and prevent bacterial overgrowth (Vantrappen et al. 1977). Its absence is associated with gastroparesis and intestinal pseudo-obstruction (Deloose et al. 2012). It has been suggested that the occurrence of MMC and its interruption by feeding involves a complex interplay between extrinsic innervation, ENS, bile acids, and peptide hormones released from different types of endocrine cells that influence the smooth muscle. The flow of chyme is determined by the movement of muscle layers which perform a complicated pattern of contractions. As the flow of gastrointestinal contents is related to the pattern of contraction, agents affecting the motility will alter the transit of GI contents. Motility studies are usually conducted in fasted state due to the presence of a well-defined motility pattern, the MMC, in comparison to a poorly defined fed pattern.

The basic functions of the GIT are similar between humans and laboratory animals. However, anatomical, hormonal, and genetic variations are translated into differences in the pharmacologically evoked motility and secretory responses. For example, in human and dog, the GI hormone motilin, which is released from the upper GIT, exerts prominent stimulatory effects on GIT motility and is involved in the initiation of MMC, whereas its function and receptor gene is lost in rodents

(He et al. 2010; Sanger et al. 2011). Differences in the role of cholinergic pathway are exemplified by lack of effect of atropine on regular occurrence of MMC in rats (Al-Saffar 1984), whereas cholinergic blockade in human (Borody et al. 1985) and dogs (El-Sharkawy et al. 1982; de Ponti et al. 1993) induces a potent inhibitory effect on MMC. Schattauer et al. (2012) demonstrated significant differences between human and rodent kappa-opioid receptors signaling that may affect the prediction of adverse effects mediated by activation of this receptor. In addition, neurokinines (NK) induce potent contractile effects via NK2 receptors in human intestine (Al-Saffar and Hellström 2001), whereas NK1 and NK3 receptors are the main contributors to smooth muscle contraction in guinea pig and rabbit (Maggi et al. 1997).

In a recent study, Keating et al. (2014) assessed the predictive potential of in vitro and in vivo rodent GI motility models by testing the effect of 15 compounds, including 8 compounds with known gastrointestinal adverse drug reactions (diarrhea or constipation). An in vitro mouse model measuring colonic peristaltic motor activity and the standard rodent charcoal meal GI transit model were found to be poor predictors of the expected motility changes associated with diarrhea or constipation observed in the clinic. Taken together, differences in functional and molecular pathways controlling GI function between rodents and human potentially compromise the translational value of drug effects from laboratory animals to humans (Sanger et al. 2011, 2013). Therefore, there is a need to develop preclinical model(s) with better predictive value for early screening of GI motility liability of novel compounds in development (Keating et al. 2014).

2.2.2 Neural and Hormonal Reflexes

Extrinsic and intrinsic nervous systems as well as local and circulating gastrointestinal hormones contribute to local and centrally mediated reflex mechanisms that regulate GI function, ingestion, secretion, mixing and propulsion, and absorption of food and defecation reflexes that force the fecal material into the rectum by intense mass movements (detailed description can be found in 'Guyton and Hall Text Book of Medical Physiology' Hall 2011).

Impulses evoked by the sight, smell, and thought of food initiate a reflex mechanism conveyed via the ANS from the hypothalamus to stimulate or inhibit local and hormonal mechanisms that regulate gastric motility and secretion. This "cephalic phase" precedes the "gastric phase" upon arrival of food in the stomach which stimulates motility and secretory reflexes via enteric and parasympathetic neurons. Gastric emptying is a complex process that involves the caloric load, intrinsic and extrinsic nervous system, and gastrointestinal hormones released in response to food intake (Hellström et al. 2006). When acidic chyme enters the duodenum from the stomach "intestinal phase," a negative feedback reflex is initiated to inhibit gastric acid secretion and slow down the gastric emptying of chyme. GI hormones secretin and CCK secreted from duodenal mucosa stimulate both exocrine and endocrine pancreatic secretion. CCK stimulates gall bladder contraction and activates local and central receptors that contribute to satiety and reduce further intake of food.

2.2.3 Intestinal Permeability and Absorption

All chemical and mechanical phases of digestion are directed toward changing the ingested material (food or xenobiotics) into absorptive forms that can pass through mucosal cells into blood and lymphatic vessels. The process of absorption occurs via passive diffusion (para and transcellular) and carrier-mediated process (facilitated or active transport). About 90 % of absorption of nutrients occurs in the small intestine.

Intestinal permeability allows for passage of molecules and ions below ~0.4 nm (MW ~250) from the lumen into the blood circulation. This "paracellular" leakage occurs through tight junctions between epithelial cells along the intestinal mucosa and varies between individuals (Piche 2014). Other molecules pass through the microvilli of the epithelial cells by "transcellular" transport. The integrity of GI mucosa is the principle determinant of nutrient and drug absorption of orally administered drugs.

The GIT has a pH gradient throughout its length which impacts on the absorption of xenobiotics. Acidic or basic drugs are absorbed under their non-ionized form which will allow the cross of biological membranes. Although the gradient is similar between species (acid in the stomach, acidic to neutral in the small intestine and basic in the colon), differences exist between species, rats, and humans being good acid excretors and dogs secreting less acid, leading to difference in solubility and therefore absorption. Feeding status also has an impact as stomach pH is higher in fed state than in fasted state (Kerns and Di 2008).

Gastric emptying time also varies between species and has an impact on the absorption, rapid emptying resulting in lower absorption of xenobiotics primarily absorbed in the stomach and earlier absorption of xenobiotics primarily absorbed in the intestine.

3 Regulatory Requirements

During preclinical development, several regulated studies are conducted; these include, but are not limited to, SP single-dose testing and repeat dose testing in toxicology studies. These studies offer an opportunity to evaluate drug effects on gastrointestinal function and structure. With respect to the preclinical investigation of gastrointestinal effects under the remit of SP, the International Conference on Harmonisation (ICH) 7A (ICH S7A) guideline recommendations state that assessing gastrointestinal function as part of the SP package is supplementary or is indicated based on the knowledge obtained about the new chemical entity (NCE) under investigation and should be performed when GI safety issues are suspected on a "cause for concern" basis (Anon 2001). GI function can be assessed by measuring "gastric secretion, gastrointestinal injury potential, bile secretion, transit time in vivo, ileal contraction in vitro, gastric pH measurement, and pooling" (Anon 2001). Gastrointestinal supplemental studies should be conducted before first time in man (FTIM) studies or during clinical development as appropriate, based on an identified cause for concern. Ultimately, gastrointestinal assessment should be

assessed prior to product approval, unless not warranted, in which case this should be justified.

The ICH S7A guideline, implemented in 2001, superseded the requirements highlighted in the "guidelines for general pharmacology studies" issued in the early 1990s (Anon 1995). The original set of guidelines defined studies that needed to be conducted to establish the overall general pharmacological profile for all test substances (category A) and studies that needed to be conducted, if necessary, in the light of the results of the studies in the category A (category B). Both categories listed studies aimed at assessing GI function. The current ICH S7A guidance provides greater flexibility for a science-driven approach in the way GI function is assessed.

The guideline recommendations for regulatory repeated-dose toxicology studies state that food intake, general behavior, body weight, hematological parameters, clinical chemistry, urinalysis, and ophthalmology should be monitored (Anon 2012). Relevant parameters should be selected to enable an identification of the toxicity profile. The parameters should be determined at relevant time points, taking the pharmacodynamics/pharmacokinetic profiles into account. In addition to final observations, these parameters should be monitored with a frequency that allows an assessment of changes over time. The selection of methodologies should be according to the current state of the art. The examinations performed during the study should also be conducted in the controls. The testing/sampling should not be performed in a way which could influence the outcome and reliability of the study. In terms of what should be measured in these studies, current regulatory guidance specifically requests routine inclusion of clinical pathology/biochemistry panels in nonclinical safety studies supporting clinical trials (Anon 2012). This is because they can be helpful to assess functional consequences of histopathological changes and because clinicians and regulators expect to see them.

4 Methods to Assess Drug Effects on Gastrointestinal Function

Models to identify drug effects on gastrointestinal function should cover gastrointestinal motility, nausea and emesis liability, secretory function (e.g., acidic, ions, hormones), and absorption aspects. Both in vitro and in vivo assessments are available and can be used either in stand-alone SP studies or as specific endpoints integrated into toxicology studies. In addition, novel in silico tools could also be considered. The choice of approach reflects the focus of a project and may involve isolated cells, organ tissues, and/or whole animals.

4.1 Assessment of Gastric Emptying and Intestinal Motility

A variety of different techniques are available for the assessment of drug-induced changes in GI motility, from the conventional organ bath preparation and the gold

standard preclinical in vivo evaluation method, the rodent charcoal meal (Harrison et al. 2004), to newer and more innovative methods including the use of wireless motility devices (Boillat et al. 2010; Boillat and Gaschen 2010) and imaging (Iwanaga et al. 1998; Chiba et al. 2000, 2002).

4.1.1 In Vitro Models

There are a variety of in vitro techniques using subcellular, cellular, tissue, or whole organ to study the pharmacological effects and mechanism of action of drugs on GIT. An advantage of in vitro studies is the ability to measure the effects of test substances on smooth muscle and ENS without the influence of external factors like systemic effect and the influence of CNS.

Current regulatory guidelines (ICH S7A) (Anon 2001) indicate that findings from "ligand binding or enzyme assay data suggesting a potential for adverse effects" should be considered in the "selection and design of safety pharmacology studies." In vitro GI functional assays could be used in parallel with in vitro profiling to maximize detecting off-target interaction and potential safety liabilities of the NCE.

Smooth muscle contraction has been studied using a number of different techniques at subcellular and cellular level (detailed review of these techniques can be found in Daniel et al. 2001). Radiotracer or fluorescent techniques can be applied to monitor calcium ion mobilization and handling by ATPase-dependent Ca^{2+} pumps present at cell membrane and sarcoplasmic reticular membrane. Electrophysiological studies using extracellular and intracellular microelectrodes have been employed to study the response to electrical stimulation or pharmacological agents. More advanced technique like the patch-clamp method is used to record, with high resolution, a single or multiple ion channel currents within the patch membrane.

Stomachs or isolated segments of GIT from small laboratory animals can be suspended in organ baths to investigate the mechanisms underlying a novel drug target and to validate physiological or pharmacological responses. Typically muscular strips or whole segments from the GIT are suspended in organ baths containing a suitable nutrient solution held at 37 °C and gassed with carbogen. GI segments from large animals and humans are usually cut in longitudinal or transverse direction parallel to muscle fiber direction, allowing longitudinal or circular muscle contraction to be studied. In some preparations, the mucosa may be peeled off or the circular and longitudinal muscle layer separated to minimize the interaction between different layers or to expose the myenteric or submucous plexus. Using conventional organ bath techniques, compounds are tested for their ability to either contract or relax GI smooth muscle using isotonic or isometric transducers.

A common technique to study the enteric nerves is by measuring changes in motility caused by electrical field stimulation (EFS). In this procedure, both sensory and motor nerves are activated in whole segments or motor nerve function in segments without mucosa and submucosa. EFS cause cholinergic-mediated contraction and nitrergically mediated relaxation. Drugs are used to study the effect and mechanism of inhibition or the potentiation of electrically evoked smooth muscle

contraction or relaxation. Many vendors have semi- or fully automated in vitro systems with software packages capable of running, in parallel, different study protocols on tissue preparations and instantly plotting the effect, efficacy, and potency of investigated compounds.

It is not yet a requirement by the regulatory authorities to incorporate data obtained using human tissue to establish GI safety profiles of candidate drugs before clinical trials, but information obtained from human tissue would be extremely useful in understanding the relevance of experimental data derived from other species. Isolated human tissue resected at the time of operation can be used immediately or after storage overnight at 4 °C in an appropriate solution without significant changes in the viability of the specimen (Bennett and Whitney 1966; Al-Saffar and Hellström 2001). However, experiments with fresh human tissue are relatively uncommon because access to human tissue can be limited and researchers have to confront issues like ethical, legal age, gender, life style, genetic background, and disease (Sanger et al. 2011, 2013). Furthermore, the use of anesthetic drugs and preoperative medications and the impact of ischemia on removed tissues for use in research are also considerations.

In vitro techniques are valuable in studying GI motility because they allow approaches that are difficult in conscious animals, though it lacks an integrated neurohormonal control system present in in vivo studies. GI human tissue preparations have the potential to reveal information related to GI safety liability of a NCE relevant to the use of drugs in clinical settings that is otherwise not possible to predict in commonly used laboratory animals due to species differences.

4.1.2 In Silico Organ Modeling

Biophysically based mathematical models related to electrophysiology have been developed on a cellular level (e.g., interstitial cell of Cajal models, GI smooth muscle models), tissue level, and whole organ level (Du et al. 2013). In addition, another field of active modeling is related to electrophysiological, electromechanical coupling, and fluid dynamics methods to predict the dynamic effects of luminal contents on GI motility, luminal content mixing, and propulsion using computational fluid dynamic (CFD) techniques (Ferrua and Singh 2010). This model showed a complex and highly three-dimensional flow profile during gastric contraction. However, findings from CFD models await further validation in relation to accurate prediction of the dynamic effects of luminal contents on GI motility. Recently, a multi-scale electrophysiological simulation model (Du et al. 2010) and CHASE software package used for cardiac simulation (Corrias et al. 2012) have been adapted to explain GI electrophysiology. As an example of a more interactive computational model, Chambers et al. (2011) incorporated the activity in intrinsic sensory neurons, excitatory and inhibitory motor neurons, and the muscle to predict muscle contraction in the guinea pig jejunum. A micro-engineering method has been employed to mimic key functional units of the human intestine, called a human "peristaltic" biomimetic gut-on-a-chip (Huh et al. 2013). In this model, human intestinal epithelial cells can be cocultured with intestinal microbes under the influence of physiological peristaltic motions

and fluid flow to recapitulate intestinal microenvironment. This biomimetic microengineering approach also opens up the possibility of integrating individual organ-on-chip models in a single instrument to recapitulate multiorgan interactions and whole-body physiology. The utility of the abovementioned in silico models await further optimization and validation before use in basic and clinical research.

4.1.3 In Vivo Models

In vivo, gastric emptying and intestinal motility are usually investigated in rodents by administering meals containing markers such as phenol red, barium sulfate ($BaSO_4$), or charcoal, subsequent to the administration of the test compound. The test meals can be used either as an indicator of liquid (phenol red) or solid transport (charcoal, $BaSO_4$). At a predefined time point, ideally corresponding to the C_{max} of the test drug, the animal is euthanized and the gastrointestinal tract removed. For gastric emptying assessment, the stomach is weighed, as its weight directly correlates with its content. Any difference in the gastric weight between treatment groups indicates altered gastric emptying.

For intestinal motility assessment, the intestines are removed (usually from duodenum to ileum) and the length of intestine filled with charcoal or $BaSO_4$ is measured and compared to the full gut length by visual inspection. The percentage of intestinal length filled by the test meal is proportional to the intestinal transit. Any difference between treatment groups indicates an alteration in the intestinal motility (increase or decrease). With phenol red, the intestinal transit is evaluated by measuring the spectral absorbance in specific subparts of the gut.

While the charcoal meal method has been shown to be relatively predictive of GI effects in the clinic (Redfern et al. 2010), the information obtained is limited (gastric emptying and small intestinal transit data only). Moreover, it is a terminal method which requires fasting prior to dosing. This makes it unsuitable to use as an additional endpoint in toxicology studies and sits best as a stand-alone SP study.

A suitable method for inclusion in toxicology studies is the fecal pellet method in rats or mice, which can be used to assess any change in the number, weight, and/or appearance of fecal pellets following test compound administration. The method has been validated with reference compounds and successfully used to determine compound-induced effects on GI transit (Bass et al. 1972; Enck and Holtman 1992; Raehal et al. 2005; Marks et al. 2013). Unlike the charcoal meal method, the fecal pellet method does not permit a detailed assessment of upper versus lower GI function, but it could still be used as an initial "flag" for a GI effect and may be a useful marker for the prediction of functional effects such as diarrhea or constipation (Marks et al. 2013). The pellet method is an easy, noninvasive technique without impact on the toxicology study endpoints. In addition, it can be performed in various experimental environments, including collection from home cage, metabolism cage, and whole-body plethysmography chambers (Marks et al. 2013).

Wireless devices such as the SmartPill® and the Bravo® capsule have been developed which can provide information on a number of GI endpoints including changes in temperature, pressure, and pH. Both technologies are minimally

invasive, as devices are orally administered to the dog and/or nonhuman primates (in fasted or fed state). Transit is wirelessly tracked through the GI tract over time. The Bravo capsule measures changes in pH only (Mahar et al. 2012), while SmartPill® can assess pH, pressure, and temperature, from which various emptying and transit times can be estimated (Boillat et al. 2010; Boillat and Gaschen 2010). These wireless techniques are widely used clinically and in the veterinary arena, but their use in pharmaceutical companies remains minimal. Limitations could be linked to the cost of the wireless devices and their supporting equipment and software, the limited amount of published preclinical data, and the large interindividual variability (Boillat et al. 2010; Boillat and Gaschen 2010; Mahar et al. 2012; Chen et al. 2008). However, wireless motility devices in general appear to be a promising technology which merits further evaluation.

Finally, imaging technology offers another noninvasive method by which GI function can be assessed, allowing inclusion in toxicology studies. Different imaging techniques (radiography or ultrasonography) are available and include the use of nonabsorbable markers such as semisolid charcoal meal, contrast medium, and radioisotope marker. A noninvasive pharmacokinetic approach measuring plasma levels of paracetamol (acetaminophen) as a marker for gastric emptying (GE) and sulfapyridine as a marker for small intestinal transit time (SITT) has been used to quantify the inhibitory and stimulatory effects of orally administered drugs on GE and SITT in conscious dogs (Sjödin et al. 2011). Imaging methods and PK profiling do not require anesthesia but only minimal restraint with preliminary acclimation, and selected time points could be chosen to fit in with toxicology study design. For example, such methods could be added to an MTD/DRF study to allow a time course assessment of GI function. Here again, the main limitations are the associated costs and availability of equipment.

4.2 Assessment of Gastric Secretion

Gastric secretion is under the control of hormonal, neuronal, and calcium-sensing receptors, resulting in causing the parietal cells to secrete concentrated acid with pH close to 1. A variety of in vitro and in vivo techniques have been used to study gastric secretion, with emphasis on acid secretion, in isolated parietal cells, isolated whole stomach preparation, or the use of recent technology of noninvasive in vivo telemetry methods.

4.2.1 In Vitro Models

Pharmacological studies on acid secretion can be performed on mucosal cell preparation from stomach mucosa obtained by enzyme dispersion and separated by centrifugation to separate parietal cells (H^+ secreting cells) and histamine secreting cells, ECL cells (Brenna and Waldum 1991).

Whole-organ study in totally isolated vascularized perfused rat stomach was described by Kleveland et al. (1987) to measure H^+ concentration and pepsin secretion in the gastric effluent. This technique allows for drug infusion through

the vasculature and direct measurement of gastric secretion using minimal amount of substance to construct a dose-response curve.

4.2.2 In Vivo Models

Gastric secretion can be assessed in anesthetized or conscious animals. In anesthetized animals, a simple, acute model involves ligating the pylorus about 1 h after administration of the test compound. A few hours later, the stomach is removed, its contents centrifuged, and the supernatant analyzed for pH or other components. This technique does not permit the evaluation of chronic treatment and may be biased by the anesthesia. Gastric fistula models have been extensively used in small animals (rats, guinea pigs, rabbits) and dogs for decades. Briefly, a cannula is placed into the body of the stomach, sutured and exteriorized so that gastric secretions can be collected in conscious, restrained animals. Changes in the content of gastric juice can be evaluated as well as the mechanisms of control involved in the secretion. Numerous variants of cannulas and pouches used in fistula models have been described (Wood and Cheung 1996). The same principle can be applied for the assessment of duodenal alkaline secretion, bile, or pancreatic secretions, if the cannula is inserted into the duodenum, or the bile/pancreatic duct instead of the stomach. As these models are quite invasive, they are rarely used in routine SP studies and cannot be integrated into general toxicology studies. For this latter purpose, as already mentioned in the section on GI motility, less invasive technologies are now available that involve wireless devices (such as Bravo® and Smartpill® capsules). They are able to measure pH as the capsule transits the GI tract, thereby providing a continuous, real-time, and easy estimate of gastric secretion.

4.3 Models of Nausea and Emesis

4.3.1 In Vivo Models

Emesis is a complex physiological event involving different organ systems (CNS, GI), and as a result, there is, to our knowledge, no in vitro model capable of accurately reproducing it. The emetic reflex, which is similar in all animal species able to vomit, is therefore directly assessed in conscious animal models. The assessment of nausea, which involves a certain level of subjectivity ("feeling of sickness" in humans), is more controversial in animals. Ferrets and dogs are typically considered to be the best species for the evaluation of emesis, dogs being usually more sensitive to emetic stimuli compared to humans. However, the sensitivity might differ according to the type of stimulus (Holmes et al. 2009). For example, macaques do not respond to apomorphine, while they are very responsive to cisplatin. Rats and mice are not able to vomit, but can display some behavioral reactions that can represent nausea, such as gasping or pica. In those species, conditioned taste aversion has been reported as a potential model of emesis (Limebeer et al. 2008). The musk shrew (*Suncus murinus*) has also been described as a suitable experimental model to evaluate drug-induced vomiting (Ueno

et al. 2002; Bolognini et al. 2013). However, its poor availability as laboratory animal limits its use in research and development.

Emesis evaluation in dogs, ferrets, or nonhuman primates usually consists of visual recordings of retching and vomiting events, and possibly premonitory signs such as licking, during a defined period following compound administration. The number of events and the latency are the most frequent parameters reported. Recordings can be performed manually by an operator (cage-side observations) or collected by means of video-monitoring. The latter technique allows the evaluation of late-stage emesis, such as observed with cisplatin or other compounds.

A more sophisticated technique consists of combining video-monitoring with telemetric measurement of abdominal pressure (Goineau et al. 2013). In the freely moving rat, intestinal electromyographic recordings have also been combined with abdominal pressure (Lesniewska et al. 2006) measurement and sampling of intestinal content.

4.3.2 In Silico Models

In silico approaches have been developed by some companies to predict nausea or emesis.

A nausea algorithm has been described by Parkinson et al. (2012) as a tool to flag the potential for nausea liability in drug discovery and development. The algorithm used informatics and data-mining tools to investigate plausible preclinical GI effects which may be associated with nausea and that could be of use for its prediction. The algorithm was built on data available from 86 marketed drugs. The main outcome was a confirmation that nausogenic or non-nausogenic drugs can be clearly separated based on their preclinical GI observations, with emesis, diarrhea, and salivary hypersecretion being the strongest predictors. The model was subsequently applied to 20 new NCEs and successfully predicted clinical outcome in 90 % of cases.

Another example comes from a cross-company 3Rs initiative (Animal Model Framework), in which data from rat charcoal meal studies (22 drugs), rat body weight measurements (34 drugs), and emesis data from dog cardiovascular studies (58 drugs) were investigated to determine whether individually or in combination they could predict nausea in Phase I clinical trials (Ewart et al. 2011). Data showed that taken alone, emesis in dogs, decreased gastric emptying, or body weight loss in rodents correlates with nausea in man for 15–23 % of drugs. However, an algorithm taking into account the response in at least 2 of the animal models enabled a more powerful approach (about 72 % sensitivity) to predicting nausea. Applying an integrated approach with data evaluation across multiple animal models could therefore predict the presence of nausea in man with reasonable accuracy and reduce the severity and frequency of drug-induced nausea in the clinic.

4.4 Models of Gastrointestinal Absorption

Gastrointestinal absorption is usually investigated as part of the ADME properties of a drug rather than as a potential safety concern. Indeed, good oral bioavailability is of crucial importance for the development of any new small molecule. Therefore, in vitro or in vivo GI absorption models used in drug development will evaluate how a candidate drug is absorbed by the gastrointestinal tract (in presence or absence of food) rather than how a candidate drug could impair GI absorption of other substances, although this aspect can also be partly covered through drug–drug interaction (DDI) studies. GI absorption depends on both GI mucosa permeability and GI transit rate.

In vitro and in vivo methods have also been used to assess changes in the mucosal integrity of the gastrointestinal tract (Smecuol et al. 2001; Yáñez et al. 2013).

4.4.1 In Vitro Models

Cell culture-based permeability screening models are used for the rapid assessment of intestinal permeability of drug candidates. A variety of monolayer models exist that mimic in vivo intestinal epithelium in human and exhibit several characteristics of differentiated epithelial cells. The human monolayer of colon adenocarcinoma cells (Caco-2) model has served as the gold standard to predict the intestinal absorption of orally administered drugs or measurement of flux of a marker molecule or active ion transport across epithelial cells. NSAID-induced mucosal damage has been demonstrated in gastric mucosa as assessed in the Ussing chamber (Curtis et al. 1993). Recently, a three-dimensional Caco-2 spheroid cell culture permeability assay has been suggested to represent the natural cell structure and morphology (Lee et al. 2014). The modified Ussing chamber accommodating a mounted gastrointestinal segment is commonly used to measure transmucosal potential and macromolecular flux through mucosal membrane as a marker for tissue integrity. Acute effects of drugs on healthy tissue or after experimental induction of tissue damage are utilized to study mucosal integrity (Bajka et al. 2003).

Recently, Lautenschläger et al. (2010) described a multiparameter model in isolated rat intestinal segments to allow perfusion through the mesenteric artery and the gut lumen. In this model, blood vessels, lymphatics, interstitial space, and lumen are maintained, allowing the assessment of potential effects of drugs on fluid homeostasis, barrier functions, transport mechanisms, immune responses, and gut motility in situ.

Though in vitro techniques offer an attractive approach to study the effects of drugs on GI permeability and absorption, they lack some of the structural and physiological components to replicate the in vivo situation like the mucus layer or normal blood flow and extrinsic and/or intrinsic nervous regulation.

4.4.2 In Vivo Models

There are various in vivo techniques available to assess intestinal absorption. They usually require the simultaneous sampling of intestinal content and blood, after intragastric or intraluminal administration of the compound of interest. Such sampling can be obtained either from anesthetized animals, with the disadvantage of the potential influence of anesthesia on the absorption properties of the intestine, or from conscious, instrumented animals.

In situ absorption models have been used in the anesthetized rat, with intraluminal administration of the test drug and collection of inflow and outflow of an isolated segment of the gut. In parallel, arterial blood is sampled and the disappearance rate of the substance is evaluated (Pang 2003). Numerous experimental protocols have been described, some of them including the cannulation of the mesenteric lymph duct or the bile duct, to assess lymphatic or portal drug transport.

Another example of a technique which can be used in large animals is the intestinal access port. One or several intestinal access ports can be surgically implanted in the duodenum, ileum, or colon, and after a proper recovery period, regular sampling of regional intestinal content can be easily done subcutaneously through the silicon chamber. After oral administration of the test compound, regional concentrations, together with plasma concentrations of this substance, are determined to assess its intestinal absorption. The intestinal access port can also be used to administer drugs directly into the intestine. This technique has been applied in the rabbit (Kunta et al. 2001) and the dog (Li et al. 2001).

As mentioned above, all of the models discussed are primarily used to assess the absorption of a drug by the intestinal tract. From a safety perspective, however, the most interesting question to address is whether the candidate drug itself can impair permeability and integrity of the intestinal wall due to mucosal damage. In principle, an ideal screening tool based on altered permeability would consist of a single drink containing non-radiolabeled probes that selectively measured damage in the proximal, mid, or distal gastrointestinal tract. Normally, only small molecules below 250 Da pass from the gut lumen into the circulation through tight junctions between mucosal cells (“paracellular” leakage). Other bigger molecules pass through intestinal microvilli by transcellular transport. So, if the probe ingested is detected in the urine, it indicates that there is an increased permeability due to mucosal damage. Traditionally, sugar probes such as sucrose, lactulose, mannitol (Camilleri et al. 2010), or more recently riboflavin (Resendez et al. 2015) have been used to assess damage in the GI tract. Such probes have been developed for clinical use but can also be used in animals. For example, in the rat, an oral cocktail of saccharide probes selectively destroyed at different levels of the GI tract can detect an increased permeability in a specific damaged site (stomach, proximal or distal small intestine, colon) (Meddings and Gibbons 1998).

5 Translation of Nonclinical Findings to Humans

The translation of nonclinical GI findings to humans has been reviewed recently by Valentin et al. (2009). A review of SP studies performed in Japan included 88 noncancer drugs that showed a good correlation between rodent intestinal transport and general adverse effects such as anorexia and constipation in humans (Igarashi et al. 1995). In the review of conventional toxicology studies that included histopathology of the gastrointestinal tract, Olson and colleagues (2000) showed good concordance between gastrointestinal effects in animals and humans, particularly for nonsteroidal anti-inflammatory drugs, anti-infective, and anticancer agents. In that review, large animal data was a better predictor than data obtained from rodents. The data also showed good correlation between animal toxicology studies and humans for a diverse set of 45 drugs (Olson et al. 2000) and may be a link to the fact that a large number of drugs are associated with gastrointestinal ADRs, thus increasing the sensitivity of detection level.

The rodent, dog, monkey, and human GI toxicity data also showed a strong correlation in the study of 21 anticancer drugs by Owens (1962). Surprisingly, in the study of 25 anticancer drugs by Schein et al. (1970), the dog was superior to the monkey as a predictor of adverse GI effects in humans. For example, monkeys were remarkably resistant to vomiting, an adverse event that was observed in humans with 21 of the 25 compounds. Gastrointestinal tract toxicity was a significant contributor to the remarkably good quantitative correlation of toxicity across species based on dose/body surface area for the 18 anticancer drugs studies by Freireich and colleagues (1966). This is not surprising since oncology drugs tend to be used at maximum-tolerated doses at which gastrointestinal side effects are quite common.

It has been suggested that the GI tract of dogs is highly physiologically similar to that of humans in terms of motility patterns, gastric emptying, and pH, particularly in the fasted state (Dressman 1986). This observation, coupled with the ability to use a formulation similar to that used in humans, makes the canine GI tract a most relevant model.

6 Biomarkers of Gastrointestinal Injury

As with other toxicities, the development and implementation of translational biomarkers for GI toxicity identification, risk assessment, and risk management are of extreme value. One of the major challenges in assessing GI toxicity during drug development is the limited translatability between preclinical models and the clinical setting. This challenge is increased due to the lack of appropriate preclinical models with confirmed added value to predicting GI injuries.

This section describes the various developments in the field of GI injury biomarkers with a clear focus on novel and emerging approaches that have the potential to generate a panel of GI biomarkers supporting preclinical and/or clinical investigations.

Unlike with kidney injury biomarkers, where between 2008 and 2010 the FDA, the EMA and the PMDA qualified the use of seven urinary biomarkers for GLP rat studies to support the safe conduct of early phase clinical trials, GI injury is still in its infancy with regard to discovery, development, and implementation of biomarkers. The challenges of developing biomarkers are even greater when the complexity of GI injuries is looked upon more carefully. These injuries include, but are not limited to, nausea, vomiting, ulceration, inflammation of the intestine, mucositis, altered fecal output, abdominal pain/discomfort, GI bleeding, and/or perforation which can be inflicted by various drug classes, chemical classes, or therapeutic areas.

Several potential blood, stool, and breath biomarkers have been studied for GI injuries, including stool and serum inflammatory markers and markers of small intestinal enterocyte mass and function.

Citrulline is an amino acid, which is the end product of glutamine metabolism. It is synthesized exclusively in the enterocyte of the small bowel (Lutgens et al. 2003; Rabier and Kamoun 1995). The circulating level of citrulline gives an accurate reflection of small bowel enterocyte mass and is, therefore, an accurate biomarker of functional small bowel enterocytes (Lutgens and Lambin 2007). Citrulline has been studied in the context of gastrointestinal toxicity following cancer treatment and has been found to correlate with oral mucositis and altered gut integrity following hematopoietic stem cell transplantation, maximum gut damage following total body irradiation, and gastrointestinal mucosal barrier injury in pediatric patients following chemotherapy (Blijlevens et al. 2004; van der Velden 2013; Lutgens et al. 2005; van Vliet et al. 2009). Given that this is a marker of small bowel enterocyte mass not inflammation, it may well have a role in monitoring chronic radiation-related injury to the gastrointestinal tract, which is a fibrotic, noninflammatory process. Citrulline is becoming a promising GI biomarker due to recent developments in its measurement by high-performance liquid chromatography (HPLC) and mass spectrometry (Crenn et al. 2008). John-Baptiste et al. (2012) showed that L-citrulline levels in rat plasma detected by mass spectrometry showed a significant dose- and time-dependent decrease in PAK4-inhibitor treatment groups compared with baseline consistent with an overall reduction in enterocyte mass, which manifested histologically as crypt necrosis and villus atrophy and fusion. It is important to point out that alteration in diet and gut microflora may potentially alter biochemical and metabolic pathways, leading to changes in metabolite measurements such as citrulline.

C-reactive protein (CRP) is an acute-phase protein and reflects a systemic host response. It is not specific for intestinal injury, but in the context of chronic gastrointestinal symptoms after pelvic radiotherapy, a raised CRP is associated with an increased risk of recurrent or new malignancy or an advanced colonic tubulovillous adenoma (Gibson and Bowen 2011; Foell et al. 2008; Khalid et al. 2007). There is no significant acute change in CRP during pelvic radiotherapy, and when significant acute changes in CRP have been reported, the CRP rise was not above 10 mg/L (Cengiz et al. 2001; Wedlake et al. 2008; Koc et al. 2003).

Diamine oxidase (DAO) is a highly active degradative enzyme of the polyamine metabolic pathways, catabolizes a variety of substrates including histamine and diamines, and is localized to the mature villus epithelial cells of rodent intestinal mucosa (Wolvekamp and de Bruin 1994; John-Baptiste et al. 2012). Although blood DAO activity level correlates with both DAO expression in the villi of the small intestinal mucosa and the severity of small intestinal mucosal lesions induced by anticancer drugs, DAO measurement is confounded by the fact that plasma levels rise markedly upon heparin stimulation prior to blood draws, with peak elevations between 30 and 60 min (Luk et al. 1980, 1981; Tsunooka et al. 2004).

Gastrins are a family of sequence-related carboxyamidated peptides produced by the endocrine G cells of the gastric antrum and duodenum in response to a number of digestion-associated stimuli. Increased serum gastrin levels are associated with duodenal ulcers, bacterial infections, tumors, and a variety of other causative factors associated with GI damage (John-Baptiste et al. 2012). These increased levels of serum gastrin were demonstrated in rats treated with omeprazole and ethanol-damaged gastric mucosa; in rats treated with acetic acid, which is known to induce gastric ulcers; and in dogs with chronic lymphocytic-plasmacytic enteritis compared with dogs without GI disease (Fattaha and Abdel-Rahman 2000; Garcia-Sancho et al. 2005; Sun et al. 2002).

Calprotectin and lactoferrin are proteins which are released when leukocytes become activated in response to tissue injury and are sensitive markers of intestinal inflammation (Foell et al. 2008; Konikoff and Denson 2006; Costa et al. 2005; Poullis et al. 2004). Calprotectin constitutes over half of the soluble cytosol proteins in neutrophil granulocytes and plays a central role in neutrophil defense, and its fecal level correlates well with the numbers of neutrophils infiltrating the intestinal mucosa and the overall severity of GI inflammation (John-Baptiste et al. 2012). High levels of fecal calprotectin can differentiate between patients with inflammatory or neoplastic bowel diseases and those with irritable bowel syndrome or normal volunteers (Costa et al. 2005). Several groups have studied the role of fecal calprotectin and lactoferrin as a biomarker of gastrointestinal toxicity after pelvic radiotherapy (Wedlake et al. 2008; Larsen et al. 2004, 2007; Hille et al. 2009). These fecal markers have been shown to potentially predict chronic gastrointestinal toxicity.

The effect of excessive fecal bile acids may be an unappreciated cause of chronic diarrhea in several clinical settings (Porter et al. 2003). Bile acids are minimally absorbed in the proximal small intestine, and the bile acid pool flows to the distal ileum, where the acids are reabsorbed by the enterohepatic transport system and then returned to the liver by the portal vein (John-Baptiste et al. 2012; Westergaard 2007). Remaining bile acids are excreted in feces. As a result, increases in fecal bile acids are indicative of malabsorption, which can cause diarrhea, but which still requires further validation due to lack of assay robustness.

Fecal miRNA assays are being developed as screening tools for sporadic human colon cancer and active ulcerative colitis (Ahmed et al. 2009). miR-194 has been proposed as potential biomarker as it is highly expressed in small intestine and colon, although it is not entirely specific to these regions (Godwin et al. 2010;

Hino et al. 2008; Lee et al. 2008). John-Baptiste et al. (2012) evaluated the expression of miR-194 in a rat PAK4-GI model and demonstrated a strong correlation between increases in this biomarker and histological lesions in the GI tract. Nevertheless, it was also documented that low-dosed animal and some vehicle control animals had increased expression of miR-194, indicating that this biomarker might be more suitable for transient GI injury.

The ^{13}C sucrose breath test (SBT) is utilized to report the status of health of the small intestinal villous. The test is based on the use of a selectively ^{13}C-labeled sucrose that enables a quantitative assessment of the absorptive capacity of the small intestine after ingestion of the stable isotope substrate, with an interval of collection of expired $^{13}CO_2$ of 90 min (Butler 2008). The cumulative percentage of the administered dose expired in a 90-min period is a marker of small intestinal damage and/or absorptive capacity. This level gives a quantitative indication of the status of small intestinal health, with a lower level indicating more impaired function (Clarke et al. 2006). The SBT can be used in both animal models and in cancer patients to follow time courses of gut damage and repair with different drugs (Wardill et al. 2013; Pelton et al. 2004; Tooley et al. 2006; Clarke et al. 2006; Howarth et al. 2006; Mauger et al. 2007).

Before these biomarkers are considered for implementation, additional method validation such as determination of sensitivity, specificity, accuracy, and precision of the individual assays is required. Subsequently, a correlation between the biomarker readout and a phenotypic endpoint should be sought. As the field of GI injury biomarkers is still in its infancy, additional prospective studies with multiple compounds, incorporation of additional time points (including recovery), and evaluation of assay performance characteristics are needed to accurately test the validity of these biomarkers to track GI damage.

Overall, the discovery, development, validation, and implementation of robust and predictable translational biomarkers have a strong potential to be beneficial for GI injury profiling in preclinical drug development and enable selection of treatment regimen and dose in a clinical setting. In addition, application of these biomarker assays will allow longitudinal and noninvasive monitoring of GI tract damage and repair following compound/drug treatment.

7 Conclusions

The gastrointestinal system plays various important physiological roles and allows the transformation of ingested food into nutrients absorbed and circulated into the organism through the blood. These functions occur by means of complex mechanical, neuronal, and hormonal pathways. Drug-related GI effects are very diverse and usually functional in nature. The most common GI signs are nausea and vomiting, diarrhea, constipation, and gastric ulceration. Despite being generally not life-threatening, they can greatly affect the quality of life, patient compliance, or be dose-limiting in preclinical studies. Therefore, there is a real need for improved preclinical GI screening of candidate drugs. Various in vitro and in vivo models are

available to identify drug effects on gastrointestinal function and assess GI motility, nausea and emesis liability, secretory function (mainly gastric secretion), and absorption aspects. In silico approaches begin to be developed, but require further optimization and validation before routine implementation in drug development. Finally, several potential biomarkers have been evaluated for predicting GI injuries, including stool and serum inflammatory markers and markers of small intestinal enterocyte mass and function. However, none of them have been qualified so far by regulatory agencies, and will need further investigation to better understand their relevance and predictive value for GIT injuries.

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Renal Safety Pharmacology in Drug Discovery and Development

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Contents

Abstract

The kidney is a complex excretory organ playing a crucial role in various physiological processes such as fluid and electrolyte balance, control of blood pressure, removal of waste products, and drug disposition. Drug-induced kidney injury (DIKI) remains a significant cause of candidate drug attrition during drug

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development. However, the incidence of renal toxicities in preclinical studies is low, and the mechanisms by which drugs induce kidney injury are still poorly understood. Although some in vitro investigational tools have been developed, the in vivo assessment of renal function remains the most widely used methodology to identify DIKI. Stand-alone safety pharmacology studies usually include assessment of glomerular and hemodynamic function, coupled with urine and plasma analyses. However, as renal function is not part of the ICH S7A core battery, such studies are not routinely conducted by pharmaceutical companies. The most common approach consists in integrating renal/urinary measurements in repeat-dose toxicity studies. In addition to the standard analyses and histopathological examination of kidneys, novel promising urinary biomarkers have emerged over the last decade, offering greater sensitivity and specificity than traditional renal parameters. Seven of these biomarkers have been qualified by regulatory agencies for use in rat toxicity studies.

Keywords
Biomarkers • DIKI • Kidney • Safety pharmacology

List of Abbreviations

ADH	Antidiuretic Hormone
ADRs	Adverse Drug Reactions
ANP	Atrial Natriuretic Peptide
AKI	Acute Kidney Injury
BUN	Blood Urea Nitrogen
CLU	Clusterin
COX	Cyclooxygenase
CysC	Cystatin C
DIAKT	Drug-Induced Acute Kidney Tubular
DIKI	Drug-Induced Kidney Injury
EMA	European Medicines Agency
eRPF	effective Renal Plasma Flow
FDA	Food and Drug Administration
FE	Fractional Excretion
FITC	Fluorescein Isothiocyanate
FTIM	First Time In Man
GFR	Glomerular Filtration Rate
GLP	Good Laboratory Practice
ICH	International Conference on Harmonisation
KIM-1	Kidney Injury Molecule-1
MAP	Mean Arterial Pressure
2-MPT	2-(a-Mannopyranosyl)-L-tryptophan
NCE	New Chemical Entity

NIC	Noninvasive Clearance
NHP	Nonhuman Primate
NSAID	Nonsteroidal Anti-inflammatory Drug
OECD	Organisation for Economic Co-operation and Development
PAH	para-Aminohippuric Acid
NCE	New Chemical Entity
PMDA	Pharmaceuticals and Medical Devices Agency
RBF	Renal Blood Flow
RVR	Renal Vascular Resistance
SCr	Serum Creatinine
SP	Safety Pharmacology
TFF	Trefoil Factor

1 Introduction: Impact of Drug-Induced Kidney Injury on Drug Development

Although the reasons for drug attrition have evolved over the years, the safety of new chemical entities (NCEs), both preclinically and clinically, remains the major cause of attrition during development, accounting for approximately 35–40 % of all drug discontinuations (Kola and Landis 2004; Kennedy 1997; Lasser et al. 2002; Cook et al. 2014). However, of more concern is the fact that over recent years, despite an increased preclinical safety testing battery, as required by regulatory agencies, there is no clear trend toward a reduction of the attrition due to safety reasons (Arrowsmith 2011; Arrowsmith and Miller 2013).

The kidney is a complex and crucial excretory organ that plays an important role in numerous regulatory processes that include fluid and electrolyte balance (i.e., ultrafiltration, reabsorption, and secretion), control of blood pressure and volume, acid–base balance, and removal of waste products. It also has a significant endocrine function (Stockham and Scott 2008) and plays an important role in drug disposition, i.e., the absorption, distribution, metabolism, and excretion (ADME) of drugs.

Unsurprisingly, drug-induced kidney injury (DIKI) remains a significant cause of candidate compound attrition in drug development (Garrett and Workman 1999; Lesco and Atkinson 2001). However, in contrast to the cardiovascular system, the safety attrition related to the renal system in preclinical development is small (2 vs. 27 %; Car 2006; Redfern et al. 2010). Furthermore there has been no link reported between serious adverse drug reactions (ADRs) and kidney toxicity in phase I clinical trials (Sibille et al. 1998) indicating that the incidence and impact of renal toxicities in preclinical development and early clinical trials are low, monitorable, and manageable (Sibille et al. 2010). This is not surprising since the kidney has a high reserve capacity so that it takes significant injury before it begins to fail. However, DIKI-related attrition rises to ~9 % in clinical development (Olson

et al. 2000), with 2 % of serious ADRs being attributed to renal toxicity (Budnitz et al. 2006; Redfern et al. 2010). DIKI or nephrotoxicity accounts for approximately 19–25 % of all cases of acute kidney injury (AKI) in clinical practice (Liano and Pascual 1996; Mehta et al. 2004). However, there have been no reports of drug withdrawal from the market due to nephrotoxicity (Stevens and Baker 2009). These observations suggest that the current nonclinical testing paradigm may not be sufficient to predict DIKI when drugs are administered in large patient populations for long periods of time. Although the majority of DIKI is associated with NCEs (small molecules), there were a few cases of renal toxicity reported in patients treated with monoclonal antibodies (Sasaki et al. 2013; Perazella 2012; Herlitz et al. 2012).

The mechanisms by which drugs produce acute and/or chronic kidney injury are poorly understood and currently histopathology is considered by toxicology pathologists to be the sole "gold standard" by which DIKI is established. However, recent developments in the biomarker field are promising, and further learnings remain to be made with respect to the relationship of the time course of the appearance of the DIKI biomarkers with respect to histopathology. As summarized in Table 1, renal ADRs or kidney toxicities are diverse and in many cases independent from the primary pharmacological target, the therapeutic indication, or the chemical class, thus making the nonclinical evaluation of DIKI more complex and challenging.

Table 1 Some drug classes that have renal side effects in clinical use

Therapeutic class	Most notable examples	Side effect
Analgesics	NSAIDs, acetaminophen, aspirin	Acute/chronic interstitial nephritis
Antidepressants	Amitriptyline, doxepin, fluoxetine	Rhabdomyolysis
Antihistamines	Diphenhydramine, doxylamine	Rhabdomyolysis
Antimicrobials	Aminoglycosides, beta-lactams, vancomycin	Acute interstitial nephritis, tubular cell toxicity, glomerulonephritis
Antiretroviral agents	Acyclovir, adefovir, tenofovir, indinavir	Acute interstitial nephritis, tubular cell toxicity
Cardiovascular agents	ACE inhibitors, clopidogrel, statins	Altered intraglomerular hemodynamics, rhabdomyolysis
Chemotherapeutics	Cisplatin, carmustine, interferon-alpha	Acute/chronic interstitial nephritis, tubular cell toxicity
Chemotherapeutics	Methotrexate	Glomerulonephritis
Diuretics	Loops, thiazides, triamterene	Acute interstitial nephritis, crystal nephropathy
Contrast dyes	Iodine or barium compounds	Tubular cell toxicity

NSAIDs nonsteroidal anti-inflammatory drugs, *ACE* angiotensin-converting enzyme

2 Anatomy and Physiology

Located in the abdominal cavity in the paravertebral gutter, the kidneys are paired organs. At the interface of the blood circulation and the urinary excretion system, the kidney is, with the liver and lungs, one of the major filters of the organism but also takes part in multiple functions such as hormonal secretion and homeostasis.

2.1 Kidney Gross Structure

The kidney is a bean-shaped organ surrounded by a capsule. In all the mammalians examined in this chapter (i.e., mouse, rat, guinea pig, dog, pig, nonhuman primate, and human), the kidney is smooth and unilobar, although, in humans, this organ is multilobar in the fetus, cortical lobation being fused rapidly after birth. The renal hilum, located at the concave surface, is the entry point of the renal artery and exit point of the renal vein and ureter.

A section of the organ reveals an external cortex (granular dark brown-red) and an internal medulla (clearer and striated) which can be discernible to the naked eye (Fig. 1).

The cortex forms a cap over the medulla, which is constituted of pyramid(s), separated by projections of cortex substance (column(s) of Bertin). The tip of the pyramid is called the papilla and empties into a calyx which itself empties into the renal pelvis becoming the ureter. The medulla can therefore be divided into outer (pyramids) and inner medulla (papilla(e) and renal pelvis), the former being itself divided into outer and inner stripes. In rodents, dogs, and monkeys, kidneys are unipyramidal (Fig. 1a); renal columns are very limited or absent and pyramids merge in a uniform medulla layer and renal papillae are fused to form a unique common papilla (renal crest) which extends directly into the renal pelvis. In humans, the presence of several pyramids and papillae (Fig. 1b, c) can be regarded as adaptation of larger body size. The inner medulla is more developed in species with high urine-concentrating capacity.

2.2 Kidney Microscopic Structure

2.2.1 Nephron: Functional Unit of the Kidney (Fig. 2)

Renal Corpuscle: Filtering Unit

Located in the cortex of the kidney, the renal corpuscle is the first section of the kidney and is constituted of the glomerulus and the Bowman's capsule. The glomerulus is a capillary network between an afferent and an efferent artery. The blood is filtered through the capillaries into the Bowman's capsule, which empties into the renal proximal tubule.

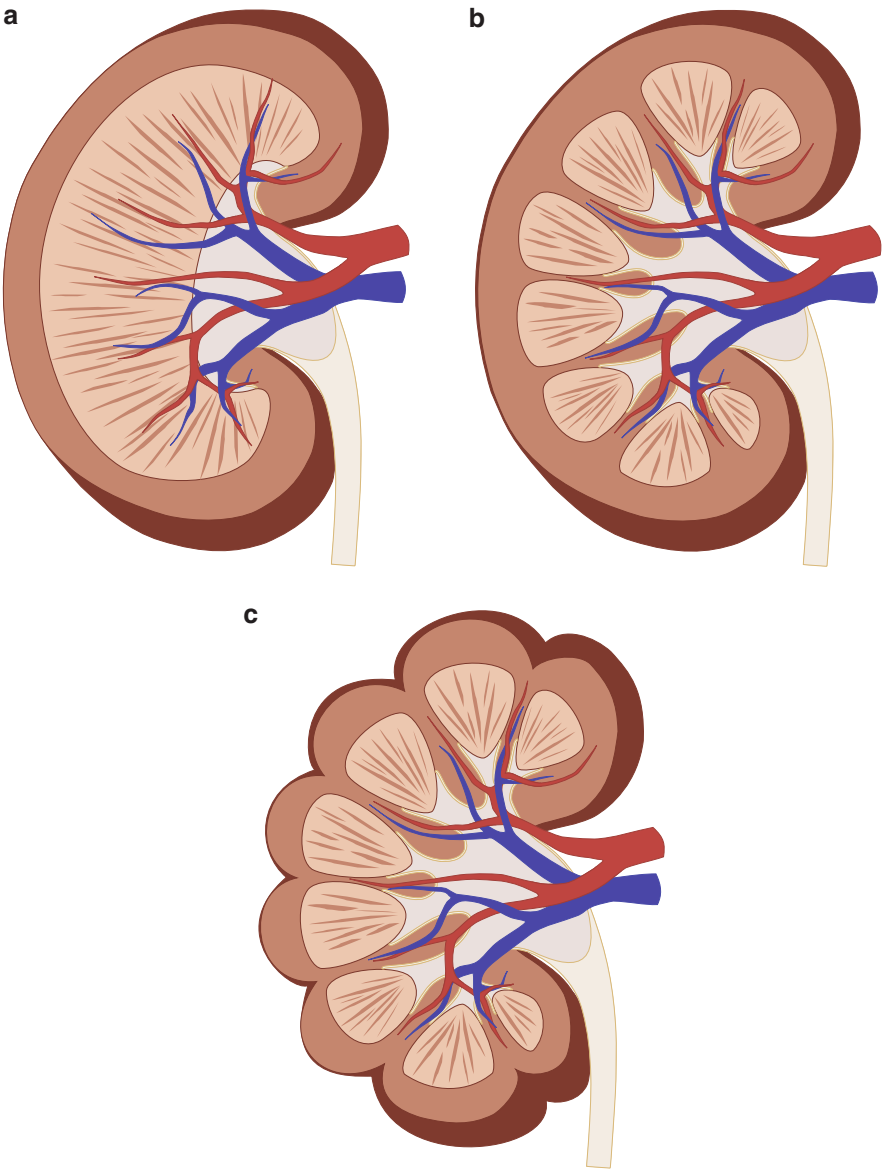

Fig. 1 (**a**) Unipyramidal, multilobar kidney section (rodents, dogs, monkeys). (**b**) Multipyramidal, unilobar kidney section (humans). (**c**) Multipyramidal, multilobar kidney section (human fetus, pigs)

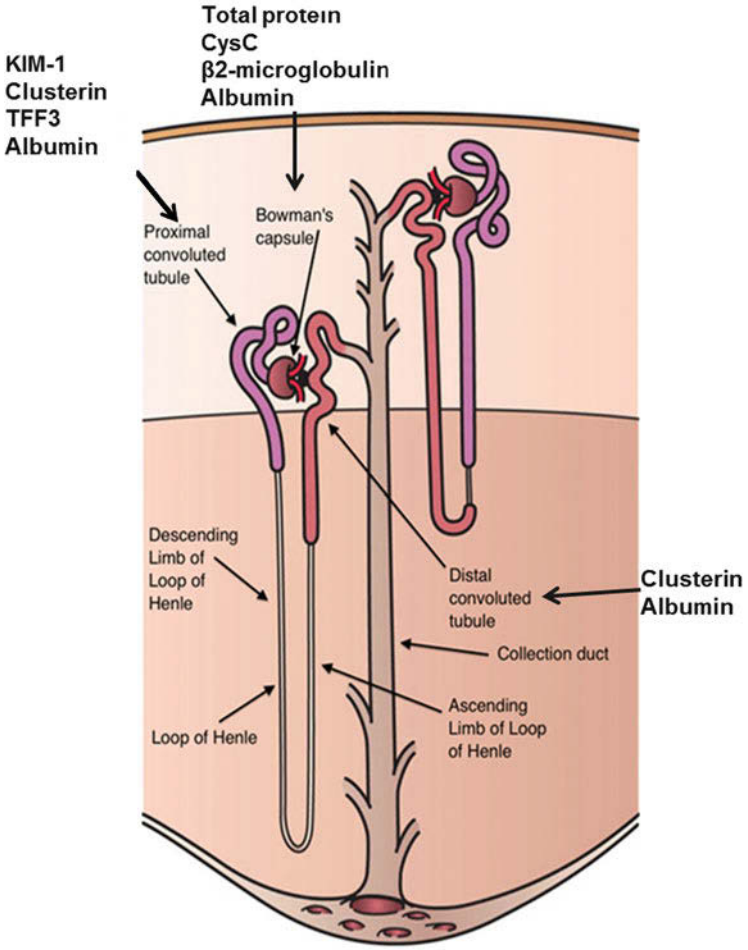

Fig. 2 Schematic representation of a nephron and qualified DIKI biomarker

Renal Tubule: Reabsorption Unit

The renal tubule extends between the Bowman's capsule and the collecting ducts. It has distinct sections which can be differentiated histologically (Fig. 2): the proximal tubule with an initial convoluted portion followed by a straight (descending) portion; the loop of Henle consisting of a descending limb and an ascending limb, the latter being divided in a thin portion and a thick portion (also known as distal straight tubule); and finally the distal convoluted tubule.

Proximal and distal convoluted tubules are located in the cortex, close to their corresponding corpuscle, whereas the straight sections pass through the cortex into the renal medulla before bending and returning to the cortex. The length of the loop of Henle varies, with subcapsular nephrons having very short loop and

juxtamedullary nephrons extending deep into the papilla. Dogs only have long loops, whereas long loops represent only 28 % of the loops in rodents.

2.2.2 Juxtaglomerular Apparatus: Regulation Unit

Located at the vascular pole of the glomerulus, where a portion of the distal nephron comes into contact with its parent glomerulus, the juxtaglomerular apparatus consists of three components: the macula densa (i.e., thickening of the epithelium of the distal convoluted tubule), the smooth muscle cell of the afferent arteriole (i.e., juxtaglomerular cells, renin-producing cells), and the extraglomerular mesangial cells.

2.2.3 Collecting Ducts: Excretion Unit

Distal convoluted tubules, through connecting tubules, flow into collecting ducts which connect the nephron to the ureter by extending from the cortex to the tip of the papilla.

2.2.4 Vascularization

The renal artery, arising from the aorta, enters the kidney through the hilum and then divides into several branches (interlobar arteries) to reach the corticomedullary junction where they give rise to arcuate arteries coursing parallel to the capsule. Two types of arteries then originate from these arcuate arteries: those irrigating the cortex (interlobular arteries) and those irrigating the medulla (descending vasa recta). Afferent arterioles of the corpuscle arise from the interlobular arteries, divide into a capillary network (glomerulus), and then reunite to form the efferent arterioles before forming again a capillary bed around the nephron. Veins run parallel to the arterial system.

2.3 Physiology

2.3.1 Excretion Role

Glomerular Filtration

Blood is filtered through the capillary walls of the glomerulus by a process of ultrafiltration driven by Starling forces (i.e., oncotic and hydrostatic forces). Water and small molecules (i.e., electrolytes, glucose, urea, creatinine, amino acids, etc.) enter the Bowman's space while larger molecules such as albumin are retained; the resulting isotonic fluid is called glomerular filtrate. Other parameters such as configuration and charge of the molecules also play a major role in ultrafiltration such that excretion of cationic molecules is facilitated.

The glomerular filtration rate (GFR) is therefore highly dependent on the capillary integrity and renal blood or plasma flow and is an important index of renal function. The way to measure GFR is described in the Methods section. For endogenous or exogenous substances that are neither reabsorbed nor secreted by the tubule, GFR (mL/min) is equivalent to clearance and corresponds to the volume of

Table 2 Comparison of glomerular filtration rate (GFR) in different species

Species	GFR in mL/min per 100 g body weight
Mouse	0.8
Rat	0.8–1
Pig	0.1–0.2
Dog	0.3
Monkey	0.2–0.4
Human	0.15–0.2

plasma from which the substance is extracted and excreted into the urine per minute, according to the formula:

$$\frac{\text{urinary concentration } \left(\frac{\text{mg}}{\text{mL}}\right) \times \text{urinary volume } \left(\frac{\text{mL}}{\text{min}}\right)}{\text{plasma concentration } \left(\frac{\text{mg}}{\text{mL}}\right)}$$

GFR is generally inversely correlated with body mass or body surface so that the rodents (Field et al. 1991) have the highest GFR, whereas that of humans is relatively low (Donadio et al. 1997; Fleck 1999) (Table 2).

GFR is also regulated by sympathetic nerve fibers which innervate arterioles of the glomerulus and by several hormones (angiotensin II, atrial natriuretic peptide (ANP), nitric oxide, endothelin, prostaglandins).

Tubular Reabsorption and Secretion

Glomerular filtrate (i.e., primary urine) is very different from the excreted urine. At each segment of the nephron, passive or active mechanisms will modify the composition of the ultrafiltrate. How the various components of the glomerular filtrate are reabsorbed is detailed below.

Reabsorption of most sodium occurs in the proximal tubule, irrespective of body needs. This reabsorption is performed through an active transport mediated by Na^+K^+ATPase, followed by a passive reabsorption of water and chloride.

The transport of water and sodium in the loop of Henle is passive and follows selective permeability. The descending limb of the loop of Henle is highly permeable to water and low to sodium; the osmolarity of the tubular urine increases with the osmolarity gradient of the interstitium (lower in the corticomedullary junction than in the inner medulla). In opposition, the ascending limb of Henle is highly permeable to sodium and low to water so that the osmolarity of tubular urine decreases in this portion. The ability to concentrate urine is highly dependent on the length of the descending and ascending branches and is more marked in rodents (Bankir 1985).

In the distal portion of the nephron (sodium) and collecting ducts (water), reabsorption is facultative, dependent on body needs and secondary to hormonal stimulation (aldosterone for sodium and antidiuretic hormone (ADH, vasopressin) for water). The atrial natriuretic peptide (ANP) also plays a role by decreasing sodium reabsorption.

Potassium (K^+) is the major intracellular electrolyte and its concentration in the body is regulated by the kidney although corrections of disturbances in potassium balance require several hours (Giebisch 1998). Whereas a marked decrease is required (20–40 %) to induce reabsorption in the kidney, an increase results in a prompt rise in K^+ clearance. The reabsorption of most K^+ occurs in the proximal tubule by passive transport, while secretion (active and passive) occurs in the distal tubule and collecting ducts. However, depending on body needs, secretion can be replaced by reabsorption.

Factors that influence the reabsorption and secretion of potassium are distal sodium supply, plasma K^+ concentration, and tubule flow rate. Renal blood flow by activation of the renin–angiotensin–aldosterone system plays also a major role.

Magnesium, calcium, and phosphate (Mg, Ca, and PO_4, respectively) concentrations are partially regulated by the kidney. These electrolytes are passively reabsorbed mainly in the proximal tubule and loop of Henle, but fine-tuning by active transporters takes place in the distal convoluted tubule (de Baaij and Bindels 2012). The reabsorption/excretion of Mg, Ca, and PO_4 is influenced by the parathyroid hormone (PTH) which also activates vitamin D in the kidney. The effects of these hormones are counterbalanced by those of the peptide hormone, calcitonin.

The amount of glucose which is reabsorbed in the proximal tubule through an active transport mechanism is proportionate to the amount filtered providing that plasma concentration remains below a determined threshold.

In normal conditions, proteins, as well as small molecules bound to plasma proteins, are not filtered by the kidneys but remain in the plasma. Proteins of low molecular weight that are filtered through the glomerulus are reabsorbed in the proximal tubule. Therefore, marked proteinuria is indicative of glomerular disease, while low-level proteinuria indicates tubular damage or early-stage glomerular injury.

Endogenous acid production (H^+), and thus control of acid–base balance, is regulated by the kidney through three mechanisms, i.e., the production of NH_4^+ ions and reabsorption of filtered bicarbonate in the proximal tubule and the buffering of hydrogen ion secretion by ammonia in the collecting tubule.

Nitrogenous waste products consisting of urea, creatinine, and ammonia ions are excreted in the urine. The elimination of drugs by the kidney occurs by passive glomerular filtration or active tubular secretion through solute transporter mechanisms (i.e., OCT, OAT, MRP, MDR, etc.). This elimination of xenobiotics is affected by their physical properties and polarity, plasma protein binding (unbound drug remaining in the plasma), and degree of saturation of transporters. Small non-lipophilic ionized substances remain in the urine and leave the body, but other substances can be reabsorbed and reenter the blood circulation through passive or active transports. Biochemical processes convert lipophilic substances into metabolites that can be excreted and therefore reduce the nephrotoxicity (Lock 1998). These biotransformation reactions involve phase I reactions (via hydrolases, oxidases, reductases, etc.) and phase II reactions (i.e., conjugation to polar compounds).

2.3.2 Regulation of Blood Pressure

Approximately 25 % of the cardiac output circulates through the kidney, which is the greatest rate of blood flow for any organ. The kidney plays a major role in the regulation of blood pressure through the secretion of renin. There are three main causes of renin release: (1) decreased blood pressure perceived by the baroreceptors situated in the juxtaglomerular cells of the afferent arteriole; (2) decreased Na+ sodium concentration in the distal convoluted tubule following a decreased blood pressure, perceived by the macula densa cells acting as chemoreceptors; and (3) increased sympathetic nervous system activity.

Renin, secreted in blood by juxtaglomerular cells, hydrolyzes a peptide (angiotensinogen) in angiotensin I further cleaved in the lungs in an active peptide, the angiotensin II. This peptide increases blood pressure through two different mechanisms: constriction of smooth muscle of the vessels and stimulation of aldosterone release by the adrenal glands. Aldosterone is responsible for the reabsorption of water and sodium which increases blood volume and therefore blood pressure. Vasopressin, secreted by the pituitary gland in response to reduced blood volume and increased plasma osmolarity, is responsible for the reabsorption of water in the collecting duct and therefore increased blood pressure.

2.3.3 Endocrine Role

The kidney produces several hormones and also controls hormones generated by other organs. The former ones are described below; the latter were already mentioned earlier in this chapter (i.e., aldosterone, ADH, ANP, and renin).

Erythropoietin is produced by peritubular interstitial cells under hypoxic conditions to increase the production of red blood cells. Its secretion is regulated by a feedback mechanism.

Most cells of the kidney possess the cyclooxygenase enzymes (COX-1, COX-2) involved in the production of prostaglandins and thromboxanes from the arachidonic acid.

Present in food and synthesized from cholesterol under its inactive form, vitamin D is involved in calcium metabolism once activated. This activation is performed in the proximal tubules.

Therefore, the kidneys receive 25 % of cardiac output and filter large volumes of plasma. As the filtrate moves along the nephron, its components are concentrated greater than threefold in the proximal tubule and greater than 100-fold in the distal tubule and the collecting ducts. As a result, the renal tubular and interstitial cells are exposed to high concentrations of medications and their metabolites, which predispose the kidneys to injury (Choudhury and Ahmed 2006).

3 Regulatory Requirements

During preclinical development, several regulated studies are conducted; those include, but are not limited to, safety pharmacology single-dose testing and repeat-dose testing in toxicology studies. These studies offer an opportunity to

evaluate drug effect on kidney function and structure. With respect to the preclinical investigation of renal effects under the remit of safety pharmacology (SP), the International Conference on Harmonisation (ICH) 7A guideline recommendations state that testing of renal function by measuring urine volume and electrolyte excretion in rats or dogs, as part of the safety pharmacology package, is supplementary or is indicated based on the knowledge obtained about the new chemical entity (NCE) under test and should be performed when renal safety issues are suspected on a "cause for concern" basis (Anon. 2001). *Urinary volume, specific gravity, osmolarity, pH, fluid/electrolyte balance, proteins, cytology, and blood chemistry determinations such as blood urea nitrogen, creatinine and plasma proteins can be used* (Anon. 2001). Renal supplemental studies should be conducted before first-time-in-man (FTIM) studies or during clinical development as appropriate, based on an identified cause for concern. Ultimately, renal assessment should be assessed prior to product approval, unless not warranted, in which case this should be justified.

The guideline recommendations for regulatory repeated-dose toxicology studies state that food intake, general behavior, body weight, hematological parameters, clinical chemistry, urinalysis, and ophthalmology should be monitored (Anon. 2012). Relevant parameters should be selected to enable an identification of the toxicity profile. The parameters should be determined at relevant time points, taking the pharmacodynamic/pharmacokinetic profiles into account. In addition to final observations, these parameters should be monitored with a frequency that allows an assessment of changes over time. The selection of methodologies should be according to the current state of the art. The examinations performed during the study should also be performed in the controls. The testing/sampling should not be performed in a way which could influence the outcome and reliability of the study. In terms of what should be measured in these studies, current regulatory guidance specifically requests routine inclusion of serum creatinine (SCr) and blood urea nitrogen (BUN) in the clinical pathology/biochemistry panels in nonclinical safety studies supporting clinical trials (Anon. 2012). This is because they can be helpful to assess functional consequences of histopathological changes and because clinicians and regulators expect to see them.

In addition, over recent years, a number of novel urinary biomarkers have emerged in rodents that have been shown to be of greater sensitivity and specificity than the traditional plasma/serum markers (Ferguson et al. 2008; Ozer et al. 2010; Yu et al. 2010; Rouse et al. 2011). Seven urinary biomarkers have been qualified by the Food and Drug Administration (FDA; June 2008), European Medicines Agency (EMA; June 2008), and Pharmaceuticals and Medical Devices Agency (PMDA; 2010) for use in good laboratory practice (GLP)-compliant rat studies to support the safe conduct of early-phase clinical trials, namely, kidney injury molecule-1 (KIM-1), trefoil factor-3 (TFF3), clusterin (CLU), ß2-microglobulin, cystatin C (CysC), albumin, and total protein (Dieterle et al. 2010a). Their appearance or excretion in urine offers the promise of greater sensitivity over functional kidney biomarkers and greater utility to detect early stages of drug-induced kidney stress, before histopathologically defined DIKI has occurred, although the use of

biomarkers in drug development and regulatory decision is still a matter of debate (Lesco and Atkinson 2001). These aspects will be covered in detail in the biomarkers and current practice sections of the chapter.

4 Methods to Assess Drug Effects on Renal Function

Models to identify drug effects on renal function should cover excretory, hemodynamic, and endocrine aspects. Although in vivo renal assessments remain more frequently used either as stand-alone safety pharmacology studies or as specific endpoints integrated in toxicology studies, investigational in vitro tools have been developed which offer good alternatives or complementary approaches to in vivo models for screening or mechanistic purposes. Before selecting a model to address a specific question, one should consider the technical feasibility and available set of validation data, the study design constraints, the interspecies differences in terms of anatomy and physiology, as well as the translational relevance of the preclinical endpoints.

4.1 In Vivo Mammalian Models

Rats, dogs, monkeys, and (mini-)pigs are the most common species used to assess both excretory function and hemodynamic function of the kidneys. Renal function assessment is mainly based on the analysis of urine and/or plasma samples and is ideally conducted in conscious, freely moving animals, to better mimic physiological conditions. Urine collection for quantitative analysis requires either urinary bladder catheterization (feasible in large animals only) or use of metabolism cages (in rodents mainly but also sometimes used for dogs and NHP) allowing urine volume determination and collection of fractions over defined periods of time (e.g., 0–8 h, 8–24 h, etc.). Such cages are equipped with a urine/feces separator. Special metabolism cages have been developed to allow simultaneous urine collection and automated blood sampling from the same animal, hence reducing the stress due to repeated handling (Han et al. 2013). They can even be coupled with telemetric system for measurement of cardiovascular function (Litwin et al. 2011; Chen et al. 2013).

While tubular function can be addressed by fractional excretion of electrolytes or by determination of urinary levels of glucose, protein, enzyme, or other newer biomarkers, GFR is usually considered as the best quantitative measurement of the overall renal function, because it is directly related to the functional renal mass. This parameter is therefore a good indicator of glomerular function and of the progress of a renal injury. Urinalysis can be easily integrated in routine toxicology studies as additional renal endpoints, but hemodynamic endpoints and GFR assessment (in particular if an exogenous marker of clearance is used), as described here below, usually requires stand-alone safety pharmacology studies (Redfern et al. 2013).

4.1.1 Glomerular Function

The GFR can be estimated by clearance measurements of endogenous or exogenous small molecules (urea, creatinine, 2-MPT, inulin, cystatin C, iohexol, or iodixanol). An ideal marker of GFR should be primarily excreted by the kidneys, freely filtered by the glomerulus, and neither secreted nor reabsorbed by the tubule. It should also be supplied to the plasma at a constant rate and exhibit no or minimal protein binding. If these criteria are met, such as for inulin, the GFR is equivalent to the renal/urinary clearance of the substance, as defined earlier in this chapter.

Clearance assessment is usually based on parallel analysis of plasma and urine samples. However, in the cases where a substance is only cleared by the kidney, the renal clearance is equal to the plasma clearance and hence, can be calculated solely based on the plasma concentration of the marker at different time points (Table 3). The major advantage is that no urine collection is needed. As an example, Katayama et al. (2010) developed a method to calculate the GFR in conscious rats using a bolus of iodixanol and a single blood sample. This method allows the repeated use of the same animals without urine collection and radioisotopes. Indeed, clearance of radioisotopes requires more sophisticated techniques such as renal scintigraphy, for which special expertise and equipment are needed, therefore limiting its use in standard safety pharmacology studies. Recently, a novel device has been developed that allows GFR measurements in conscious laboratory animals without the need for blood or urine sampling or laboratory assays (Schock-Kusch et al. 2011; Schreiber et al. 2012). The noninvasive clearance (NIC)-kidney device is a miniaturized detection device weighing 3.2 g that when mounted on the back of laboratory animals enables the transcutaneous measurement of the elimination kinetics of the fluorescent renal marker FITC-sinistrin (fluorescein isothiocyanate 1-sinistrin; active pharmaceutical ingredient of the commercially available GFR marker Inutest®) in conscious animals. This allows the measurement of the clearance of FITC-sinistrin from the plasma in real time without the need for any blood sampling and thus is a means of measuring GFR with a much lower burden on the individual animal.

4.1.2 Tubular Function

If plasma markers provide relevant information about the level of renal perfusion and functional mass, the analysis of urine allows the identification of the functional status of particular nephron segments. In general toxicology studies, routine urinalysis usually includes visual assessment of urine (color, clarity), volume, specific gravity or osmolality, pH, quantitative or semiquantitative protein, and glucose content. Dipstick test strips as used in human medicine also include the determination of other parameters, such as ketones, bilirubin, urobilinogen, hemoglobin, etc. For example, glucosuria in the absence of increased plasma glucose indicates a functional defect of the proximal tubule. Moderate proteinuria is indicative of tubular damage, while massive proteinuria indicates glomerular disease, so that this parameter is less specific. Other protein biomarkers as well as enzyme biomarkers will be discussed in the next section. As soon as a quantitative assessment of an analyte or biomarker is required, measurement of urine volume becomes

Table 3 Tests for glomerular filtration rate (GFR) assessment

Marker	Fluid collected	Species	Limitations
Urea	Plasma or serum	Human, dog, rat	Passive reabsorption in tubules, extrarenal influences (food, bleeding, etc.), insensitivity to small decrease in renal functional mass
Creatinine	Plasma or serum	Human, dog, rat	Extrarenal influences (age, muscle mass), poor sensitivity, can be secreted by proximal tubule in rats
2-(a-Mannopyranosyl)-L-tryptophan (2-MPT) clearance	Plasma and urine	Human, rat	Less extrarenal influences, good correlation with inulin clearance
Cystatin C clearance	Plasma or serum	Human, dog, rat, mice	Affected by inflammation or neoplasia, higher sensitivity than creatinine
Endogenous creatinine clearance	Plasma and urine over 12 h or 24 h	Dog	Overestimation of GFR due to tubular secretion and non-creatinine chromogens
Exogenous creatinine clearance	Plasma with or without 12 h or 24 h urine	Dog	Subcutaneous or intravenous injection of creatinine to increase the concentration by 10x
Inulin clearance	Plasma and urine	Human, dog, rat	
Iohexol clearance	Plasma	Human, dog, rat	Stable, not toxic but requires high doses; clearance = dose/AUC
Iodixanol clearance	Plasma	Human, dog, rat	
Protein excretion	Urine	Dog	No differentiation between glomerular and tubular proteinuria

necessary. However, if complete timed urine collection cannot be conducted, urine creatinine concentration can be used to normalize the quantity of the analyte in a spot urine sample, as its daily excretion is roughly constant.

Urine electrolytes levels are highly sensitive to the functional state of the kidney. The most useful information is the fractional excretion (FE), which is the proportion of electrolyte excreted in urine from the plasma. Accurate FE calculation should be done in parallel with GFR assessment. If GFR is normal, any change in FE reflects tubular malfunction. In this case, FE will increase for electrolytes primarily reabsorbed by the kidneys and will decrease for secreted electrolytes.

4.1.3 Hemodynamic Function

Renal blood flow (RBF) can be measured either directly or indirectly. Direct measurement requires the placement of a flow probe around the renal artery, which is technically difficult in rats due to their small size. Therefore, renal hemodynamic studies are rather conducted in large animal species such as dogs, (mini-)pigs, and nonhuman primates, due to the easy surgical accessibility of

kidneys and vessels. RBF assessment is usually coupled with systemic blood pressure monitoring, using a pressure catheter placed into an artery (e.g., femoral). These probes can be exteriorized via a connector or a vascular access port. Telemetry avoids exteriorization of these probes and therefore induces less risk of infections or postoperative complications. It also allows the animals to be kept over long periods of time, and thus, they can be reused across successive studies. Another hemodynamic endpoint is the renal vascular resistance (RVR), calculated as the ratio between RBF and mean arterial pressure (MAP). RVR can be increased in case of renal dysfunction or in case of systemic hypertension.

An indirect measurement of RBF can be made using para-aminohippuric acid (PAH) clearance. This molecule is an ideal marker of the effective renal plasma flow (eRPF), as it freely filters through the glomerulus, and any amount remaining in the peritubular capillary plasma is secreted into the proximal tubule. Therefore, essentially all PAH passing through the kidneys appears in the urine. For this reason, the PAH clearance is directly proportional to the rate of plasma flow through the kidneys. If the hematocrit is known, the total renal blood flow can be easily calculated from the eRPF value.

An easy and rapid method with intravenous bolus administration of an iohexol/PAH mixture in dogs has been developed for simultaneous measurement of GFR and eRPF, based on plasma clearances of both substances (Laroute et al. 1999).

4.2 In Vivo Non-mammalian Models

The zebrafish (*Danio rerio*) larva has gained increasing interest over the last decade as an alternative to mammalian in vivo models (Redfern et al. 2008). The zebrafish kidney is genetically and morphologically close to that of mammals, except that the pronephros of larvae consists only of a fused glomerulus with one nephron on each side. While previous investigations of renal function were limited to morphological studies, new techniques have emerged more recently, which allow functional investigations, such as renal clearance and cardiovascular flow. Assessments were based on measurements of FITC-inulin intensity in the caudal artery and excreted FITC-inulin and were validated using gentamicin and high salt loading (Rider et al. 2012). Offering the possibility of high-throughput screening, visual transparency, low cost, and genetic manipulation, zebrafish are a promising and simple tool for studying renal function. However, its validation with a larger set of known nephrotoxic drugs appears necessary.

4.3 In Vitro Models

Significant progress has been made in the last two decades in the development of various in vitro techniques for the isolation and culture of kidney cells. To our knowledge, cellular assays are mainly used in mechanistic studies aimed at understanding normal and disease renal cell function, rather than as routine screening

tests to assess drug effects in safety pharmacology. Because of the diversity of the kidney structure, which contains no less than 15 different cell types in the tubular epithelium, a large variety of cell lines have been developed. They predominantly originate from human, mouse, rat, and rabbit. Most cell types can be obtained by microdissection techniques and be grown as primary culture monolayers, with limited life span (3–5 passages). Some spontaneous permanent cell lines or immortalized renal cell lines are also available, offering the advantage of a renewable proliferative source of cells but the disadvantage of some abnormal properties (Wilson 2009). These cell populations have been used to study ischemic or hypoxic injury, effects of nephrotoxic agents (environmental toxins, therapeutic agents), congenital renal diseases, or renal cancer and fibrosis. Various morphologic and biochemical parameters can be measured in cell cultures to evaluate proliferation, adhesion, polarization, and differentiation of renal cells. Morphologic analysis is complemented by a huge variety of biomarkers specific of each function and detectable by immunohistochemistry (see section on biomarkers).

Renal slice technology has been extensively exploited for pharmacology and safety assessments. This technique involves the removal of longitudinal sections of kidney tissue using a precise-cut microtome. Dog, rat, and rabbit kidneys are the most frequently used, but renal slices of human origin have started to be used more recently. Over other in vitro cellular models, renal slices offer the advantage to keep the architecture and cell heterogeneity of the whole organ, as well as surrounding interstitial and vascular elements. They are functionally able to transport ions, to maintain sodium and potassium balance, and to exhibit cell–cell interactions. They can be used for short-term (up to a few hours) or long-term (up to 48 h) incubation with test drugs. The primary endpoints of renal slices are metabolic function or morphologic analysis, which can be coupled with gene expression and biomarker analysis (Vickers et al. 2004).

Isolated perfused kidney preparations have been successfully used to study renal physiology and pharmacology. Indeed, this model enables investigation of renal clearance, uptake, transport, and metabolism of substances. It also allows hemodynamic measurements (i.e., renal blood pressure and flow, renal resistance) and evaluation of endocrine function (e.g., renin secretion). This preparation can be applied to the preclinical assessment of renal side effects of pharmaceutical drugs. Although kidneys of cats and dogs have been used in the past, the rat is now the preferred species, due to its small size and the ease of surgical techniques. However, kidneys of mice or pigs can also be used. There are two different versions: the ex vivo perfusion model, where the kidney is completely isolated from the body and maintained under controlled conditions in a perfusion chamber, or the in situ perfusion model, where the kidney remains in the body of the animal, hence preserving the hormonal regulation and innervation. The renal artery is cannulated and perfused at constant rate with a buffer solution containing all elements necessary for kidney metabolism and viability (glucose, albumin, electrolytes, amino acids, etc.). Changes in arterial pressure can be measured and effluents collected for biochemical analysis. Another variant of this model is the non-filtering kidney, in which the glomerular filtration is bypassed so that the test substance can only access

the renal tubule. This preparation is used to specifically study renal tubular absorption. In contrast, in the fixed kidney preparation, the tubular function is blocked by glutaraldehyde perfusion, allowing the evaluation of glomerular capillary wall permeability. The collected urine is a glomerular ultrafiltrate, and the kidney is equivalent to a simple filtrating membrane. Similar results can be obtained using isolated glomeruli.

More recently, organ-on-chips have emerged as exciting innovative tools combining microengineering, cell biology, and physiology approaches. Regarding the renal system, human kidney proximal tubule-on-chip has been successfully used to assess drug transport and nephrotoxicity of drugs (Jang et al. 2013). Primary epithelial cells from the proximal tubule are cultured on an extracellular porous, matrix-coated membrane, with two adjacent channels created on the device, one on the apical side mimicking the lumen and the other at the basal side representing the interstitial space. Such tool seems to more closely mimic the in vivo response of tubular cells than any other in vitro systems used so far and appears as a promising tool to investigate human renal toxicity during drug development.

4.4 In Silico Models

Several in silico models for renal excretory function have been described in the literature, focusing on the fraction of the drug excreted in urine as endpoint. They are based on the analysis of human urinary excretion data of 150–160 drugs and use a set of 72–94 physicochemical and structural descriptors to build their model (Doddareddy et al. 2006; Manga et al. 2003). Among these descriptors, lipophilicity is one the most important parameters influencing renal clearance, together with hydrogen bond donor count, presence of carboxylic acid groups, and polar surface area. The final result is a multi-categorical model where compounds are classified according to the percent of urinary excretion (from low to high). However, these models were found to have only moderate predictivity for renal clearance of new compounds (Feng et al. 2010).

The SAPHIR project (a Systems Approach for PHysiological Integration of Renal, cardiac, and respiratory functions), initiated in 2008 under the 6th European Framework Program, provides a prototype core model of human physiology targeting the short- and long-term regulation of blood pressure, body fluids, and homeostasis of the major solutes (Thomas et al. 2008). It also includes the main regulatory sensors (baro- and chemoreceptors) and nervous (autonomic control) and hormonal regulators (antidiuretic hormone, aldosterone, and angiotensin). The resulting modeling resource is an open-source quantitative kidney database available to the general community (http://physiome.ibisc.fr/qkdb). Another initiative supported by the FDA is aimed at predicting ADRs of novel drugs for specific system organ classes, using publicly available preclinical screening data from the PubChem BioAssay database (Pouliot et al. 2011). However, for renal and urinary disorders, the predictivity was not very high and remains below the fixed threshold. The best predictions were for immune disorders and blood and lymphatic system

disorders. Therefore, the use of this tool for predicting renal adverse drug reactions is not currently recommended.

5 Biomarkers of Kidney Injury

The development, validation, and implementation of translational biomarkers that allow for renal toxicity identification, risk assessment, and risk management are of extreme value. The ideal biomarker or panel of biomarkers should demonstrate the necessary sensitivity and specificity to predict toxicity in preclinical models and in a clinical setting. One of the major challenges in assessing DIKI is the limited translatability between preclinical models and the clinical setting mainly due to the suboptimal inappropriate tools currently available.

It is important to note that drugs can induce increases in SCr and BUN and decreases in GFR, for example, by inducing changes in renal perfusion pressure or by affecting the proximal tubular transport of creatinine or the reabsorption of urea, without detectable DIKI, and thus may give false-positive signals in studies evaluating renal function.

In chronic progressive renal failure, as much as 80 % of the functional renal mass can be lost before functional biomarker level rise. This means that the functional biomarkers can underestimate the actual extent of kidney injury and thus result in a false-negative signal. Therefore, while the functional biomarkers SCr and BUN may be helpful to estimate the impact of DIKI on a change, if any, on kidney function (i.e., GFR), they are not particularly useful tools for predicting DIKI potential or susceptibility before frank kidney injury has occurred.

This section describes the regulatory accepted DIKI biomarkers, their application, and the limitations that have been identified to date. It also provides an insight to novel approaches for DIKI biomarker identification, development, and validation through the combination of established and state-of-the-art approaches.

5.1 Qualified Biomarkers

Between 2008 and 2010, the FDA (2008), the EMA (2008), and the PMDA (2010) qualified the use of seven urinary biomarkers for GLP rat studies to support the safe conduct of early-phase clinical trials: KIM-1, CLU, TFF-3, β2-microglobulin, CysC, albumin, and total protein. These are detailed below.

KIM-1 is a type I cell membrane glycoprotein which contains, in its extracellular portion, a six-cysteine immunoglobulin-like domain, two N-glycosylation sites, and a T/SP-rich domain characteristic of mucin-like O-glycosylated proteins. The ectodomain of KIM-1 is shed from cells in vitro and in vivo into the urine in rodents and humans after proximal tubular kidney injury or in patients with renal cell carcinoma. KIM-1 confers on epithelial cells the ability to recognize and phagocytose dead cells that are present in the postischemic kidney and contribute to the obstruction of the tubule lumen that characterizes acute kidney injury. In

addition to the facilitation of clearance of the apoptotic debris from the tubular lumen, KIM-1 may play an important role in limiting the autoimmune response to injury since it is known in many systems that phagocytosis of apoptotic bodies is one mechanism for limiting the pro-inflammatory response (Bonventre 2009).

The secreted isoform of clusterin (CLU) is a 76–80-kDa glycosylated protein with extensive posttranslational modifications, such as glycosylation, cleavages, and dimerization. In the context of kidney injury, CLU has been suggested to play an antiapoptotic role and to be involved in cell protection, lipid recycling, cell aggregation, and cell attachment. Clusterin overexpression was shown to be induced by different types of kidney injury in glomeruli, tubules, and papillae of rats and dogs as a result of drug nephrotoxicity, surgery and ischemia, and renal diseases. Changes of protein levels of CLU have been observed in kidneys and in the urine in animal studies as well as in human. Although CLU is expressed in several tissues, its molecular size prevents a filtration in the kidney, thus rendering its urinary levels specific to kidney injury (Dieterle et al. 2010a or b, Rosenberg and Silkensen 1995; Kharasch et al. 2006; Hidaka et al. 2002).

TFF3, TFF1, and TFF2 are small peptide hormones secreted by mucus-producing cells, and by epithelial cells of multiple tissues, in mammals. By inhibiting apoptosis and promoting survival and migration of epithelial cells into lesions, TFF3 facilitates restoration of intestinal epithelium as a protective barrier against injury and plays a role in inducing airway epithelial ciliated cell differentiation. In rat kidney, Tff3 mRNA is highly expressed with binding sites in the collecting ducts (Yu et al. 2010; Madsen et al. 2007; Kinoshita et al. 2000).

β2-Microglobulin is a 12-kDa polypeptide chain that is constantly synthesized throughout the body. It is filtered by the glomeruli and nearly completely reabsorbed and catabolized in the tubules so that only 0.3 % of the filtered β2-microglobulin is found in the urine. Impairment of tubular uptake elevates urinary β2-microglobulin concentration. This occurs by increase in high-molecular-weight protein leakage resulting in a high protein load in the tubules, competing with tubular uptake of β2-microglobulin and increasing its excretion into urine, or by tubular reabsorption being directly impacted by treatment with drugs or specific tubular diseases (Dieterle et al. 2010a; Thielemans et al. 1994; Gatanaga et al. 2006).

CysC is a non-glycosylated low-molecular-weight protein of 13 kDa continuously produced by all nucleated cells. CysC is directly filtered from blood in the glomerulus, and its serum levels are an ideal estimator of the glomerular filtration rate. Virtually all filtered CysC is reabsorbed and metabolized by the tubules, and an impairment of reabsorption in proximal tubules by the same mechanisms described for urinary β2-microglobulin can lead to a significant increase of urinary levels in human and rats (Dieterle et al. 2010a; Mussap and Plebani 2004; Madero et al. 2006; Herget-Rosenthal et al. 2004).

Albumin is a major serum protein and is often the most abundant protein found in urine during renal injury. The quantity of albumin appearing in urine is very important to distinguish the etiology of renal disease. Normally, a small fraction of serum albumin (<1 out of 1,000 albumin molecules) passes the glomerular

basement membrane and is reabsorbed in the proximal tubules. Subnephrotic range albuminuria (<3.5 g/dL) may occur in renal tubular disease, such as early-stage diabetes, or in drug-induced acute kidney injury caused by several chemotherapeutics and by gentamicin antibiotics known to cause direct tubular toxicity (Yu et al. 2010; Christensen et al. 2007; Tugay et al. 2006; Kern et al. 2000).

Total urinary protein has been highlighted as a diagnostic marker and as a factor predicting progressive loss of renal function in clinical and nonclinical contexts. Increased urinary excretion of protein, typically referred to as proteinuria, results from alterations of the glomerular filtration barrier usually associated with damage of the glomerular podocytes (Dieterle et al. 2010a; D'Amico and Bazzi 2003; Shankland 2006).

The main claims of the qualification focused on the capacity of the various biomarkers to address early identification of DIKI by outperforming or complementing the established biomarkers (BUN and SCr) and on the ability of the biomarker to discriminate between tubular and glomerular alterations. Of these biomarkers, urinary KIM-1, clusterin, and albumin were shown to outperform and complement BUN and SCr as early diagnostic biomarkers of drug-induced acute kidney tubular (DIAKT) alterations in rat toxicology studies; urinary TFF3 was shown to complement BUN and SCr in the early diagnosis of DIAKT alterations; and total urinary protein, CysC, and β2-microglobulin were shown to outperform and complement BUN and SCr as early diagnostic biomarkers of drug-induced glomerular alterations of damage resulting in impairment of kidney tubular reabsorption (Dieterle et al. 2010a). These conclusions were based on the increased sensitivity and area under the receiver operating characteristic curves of the new biomarkers against BUN, SCr, and the "gold standard" histopathology scoring (Dieterle et al. 2010a, b).

It was also proposed that urinary KIM-1, albumin, total protein, CysC, and β2-microglobulin may be considered individually qualified for regulatory decision making as clinical bridging biomarkers appropriate for use in phase 1 and 2 clinical trials for monitoring kidney safety when animal toxicology findings are considered as of concern-generating.

Therefore, the qualification of this panel of seven DIKI biomarkers sets the foundations for the development and validation of other biomarkers that may focus on abnormalities of the proximal, distal, and collecting tubules and ducts or papillary injury.

The use of these biomarkers in preclinical studies should be considered in parallel with an evaluation of histopathological changes in the kidney. Histopathological changes have been well characterized in commonly used experimental animals, and they currently remain to be considered as the "gold standard" against which biomarkers from body fluids are measured. The combination of both approaches may address the shortcomings of each one of the individual approaches: the need for further implementation of the DIKI biomarkers and the inability to establish the dynamic of histopathological changes, i.e., onset, time course, and reversibility.

After the qualification of the DIKI biomarkers by regulatory agencies, a series of limitations were identified. These caveats focused mainly on the limited cross-species reproducibility and applicability of the biomarkers, the need for a better characterization of the robustness of the biomarkers according to strain and gender of the preclinical models, the technical robustness of the assays used for biomarker measurement, as well as the applicability of these biomarkers in acute, subacute, and chronic studies. To this end, various studies have been conducted to address these caveats and have provided further understanding on the robustness, reproducibility, variation, and performance of these biomarkers in preclinical models (Pinches et al. 2012; John-Baptiste et al. 2012; Harpur et al. 2011; Guha et al. 2011).

As stated by the regulatory authorities, the use of DIKI biomarkers is considered voluntary. As a result, pharmaceutical companies have applied different strategies when it comes to using them in safety studies. Indeed, in a recent survey of the top 15 pharmaceutical companies as determined by size of the R&D budget, it was shown that these markers were not shown not to be uniformly, comprehensively, or consistently used by the companies surveyed mainly because of cost, uncertainty regarding their translatability and applicability to humans, and interpretation of current biomarker signals in a risk assessment context, still requiring concurrent kidney histopathology. Indeed, KIM-1 and albumin were the only biomarkers that were used across the board (Benjamin et al. 2015).

5.2 Novel Exploratory Biomarkers

With the advent of novel biomarker strategies based on integrating established and cutting-edge approaches, DIKI can greatly benefit from the development and implementation of molecular biomarkers. In recent years, exploratory approaches have focused on untargeted and targeted mass spectrometry-based proteomics and metabolomics but have also built on the expansion of genomics and epigenomics approaches. These novel approaches, when combined with more established ones (i.e., classical clinical chemistry, functional assessment, toxicogenomics, histopathology), could potentially lead to the development of improved algorithms for predicting region-specific renal safety.

Examples of novel exploratory biomarkers and their overall scientific approaches have been increasing the last few years. Studies have demonstrated how, in preclinical models, changes to chromatin environment or microRNAs, such as miR181a and miR34a, may be indicative of proximal and distal tubular injury, how vanin-1 at the mRNA and protein level is an indicator of proximal tubular injury, how robust mRNA signatures measured in biofluids have the potential to be better predictors of region specific kidney injury, or how urinary proteomic biomarkers may serve as a valuable tool to investigate potential new drug candidates for the risk of renal safety (Bhatt et al. 2010; Zhu et al. 2012; Hosohata et al. 2011; Kondo et al. 2012; Rouse et al. 2012).

As with the seven qualified DIKI biomarkers, there will be the need for the implementation of a robust validation strategy which will be based on the

foundations from the first renal biomarker qualification submission (Dieterle et al. 2010b) and also addressing the caveats that were identified thereafter.

6 Current and Future Industry Practices with Respect to Renal Safety Pharmacology

As stated previously, the ICH S7A recommendations state that the preclinical investigation of renal effects under the remit of safety pharmacology is supplementary and should only be performed when renal safety issues are suspected on a "cause for concern" basis (Anon. 2001). Therefore, assessment of renal function might not be performed by all companies.

Indeed, a recent survey conducted within the largest 15 pharmaceutical companies, as determined by R&D size, investigated what strategies are currently used to study renal effects of candidate drugs in both ICH S7A-compliant SP studies and within general toxicology studies (Benjamin et al. 2015). The results of this survey showed that the majority of those questioned (90 %) performed renal/urinary function measurements in toxicology studies (i.e., repeat-dose studies) and that only 50 % of those surveyed stated that they perform renal SP studies (single dose) and only do so if there is a specific cause for concern. Interestingly, all the companies that perform renal SP studies also conduct renal/urinary measurements in repeat-dose toxicity studies. In terms of the species and methods used to investigate renal function, the rat, dog, and NHP were used to similar degrees. Although one respondent investigated renal function solely in the rat and the other only used large animals, all the other respondents used a combination of rat, dog, and NHP or all three species.

Thus, the results suggest that most pharmaceutical companies embrace the ICH S7A guideline philosophy with respect to renal SP studies and only perform such studies when there is an identified or suspected cause for concern. It could be postulated that the reasoning for measuring renal function after repeat dosing of compound rather than after a single dose of compound is due to the fact that the kidneys have a high level of functional reserve. Indeed, it is well known that in chronic progressive renal failure, as much as 80 % of the functional renal mass can be lost before functional biomarker levels such as SCr and BUN rise (Sieber et al. 2009). Interestingly, Redfern et al. (2010) used published reviews and BioPrint® to perform an analysis of projects terminated in clinical development across all pharmaceutical companies between 1999 and 2009. It was found that although 9 % of projects were terminated due to renal toxicity, none of these toxicities were detected by the current, standard SP one-dose renal model, but rather they were detected by histopathology in the preclinical toxicity models or at later stages in development.

It should be noted that the format of renal SP studies differs markedly from that of repeat-dose toxicology studies. A renal SP study typically consists of a single-dose administration to conscious animals (usually rat) with compound at levels up to the maximum tolerated dose. Urine is collected over 24 h (this can be split into

multiple collection periods) and plasma is also sampled pre-dose and at a set time post-dose. The ICHS7A guidance states that a renal safety pharmacology study should include the measurement of urine volume and electrolyte excretion (Anon. 2001). However, it is common for multiple analytes to be quantitatively analyzed in the plasma and the urine (see Methods to Assess Renal Function section). In contrast, the primary aim of regulatory repeat-dose toxicity studies is to identify the potential hazards of NCEs by evaluating target organ toxicities and the exposure levels at which these occur, before humans are exposed. The standard duration is 28 days ("1 month"); they are required to be performed in a rodent and a non-rodent species and are conducted according to the principles of good laboratory practice (GLP). The primary endpoints of general toxicology studies are pathological ones, that is, histopathological examination of a standard set of tissues postmortem. However, blood and urine samples are also taken from these studies and current regulatory guidance specifically requests routine inclusion of SCr and BUN (Anon. 2012). Urinary collection is limited to a single short collection period which can be over a set time period or until sufficient volume for analysis is obtained.

Therefore, the urine and plasma analysis in a general toxicity study can be less comprehensive than in a renal SP study, being limited to excretory endpoints, and it is not unusual for dipstick analysis to be used in lieu of accurate quantitative analysis. For example, hemodynamic endpoints such as GFR and eRPF are not routinely measured in general repeat-dose toxicity studies. Indeed, of the SP representatives questioned by Benjamin et al. (2015), only 20 % directly measure the hemodynamic parameter GFR (and sometimes renal blood flow), and the majority of respondents use creatinine clearance, which was found to be calculated by the majority using snapshot measurements of urinary and plasma creatinine, as a surrogate marker of GFR. This could be due to the more complex and labor-intensive nature of studies to accurately measure GFR or eRPF, with infusion of tracer and multiple blood or urine sampling that prevents these measurements being added on to toxicology studies as opposed to the relative ease of the addition of a single urinary collection period. Thus, it would appear that even though there are now integrated platforms available that can simultaneously measure renal hemodynamics (GFR and eRPF), excretory function (quantitative urine analysis including DIKI measurements), and blood chemistry all in the same animal (Chen et al. 2013), these systems are yet to be utilized routinely within the pharmaceutical industry.

The seven regulatory approved DIKI biomarkers mentioned previously are yet to be fully utilized across the pharmaceutical industry, and their regulatory approval has not impacted the design of preclinical safety studies, as determined by the surveys performed by Benjamin et al. (2015). It was found that 70 % of respondents measure DIKI biomarkers in their renal/urinary studies with KIM-1, uTP, and albumin being the only regulatory approved DIKI biomarkers that were measured by all the respondents and CysC, B2M, TFF-3, and clusterin being measured to lesser degrees. Interestingly, some other, non-regulatory approved DIKI biomarkers including osteopontin, lipocalin-2, and GST-α are also measured.

Current experience with DIKI urinary biomarkers is largely limited to well-controlled studies of known kidney toxicants such as cisplatin, and there is a paucity of DIKI biomarker data on the effects of compounds that are known to impact kidney function but not to produce DIKI (i.e., negative controls) or of compounds that are known to produce renal histopathology but to a lesser degree than compounds such as cisplatin. Therefore, it is hoped that further validation and investigation of both the regulatory approved and non-regulatory approved DIKI biomarkers will lead to further understanding of the DIKI biomarkers and thus an increase in their utilization by the pharmaceutical industry for the investigation of NCE effects on the renal system.

Thus, the following recommendations can be made, based on what is currently known about the differences between a renal SP study and the renal measurements on repeat-dose toxicity studies:

1. Repeat-dose general toxicity studies should include robust renal functional endpoints to ensure accurate quantification of renal function.
2. If a NCE has a renal liability, then DIKI biomarkers should be assessed in repeat-dose toxicology studies, which include histopathology endpoints, rather than in SP studies (Redfern et al. 2013).
3. If there is an overriding cause for concern with regard to nephrotoxicity, then a stand-alone SP renal study should be performed. This could take the form of a standard design safety pharmacology renal study or a study after single or repeat doses of compound which could include detailed assessments of renal function such as assessment of renal reserve or hemodynamic function (Valentin and Hammond 2006).

However, as stated by Redfern and colleagues (2013), regulatory general toxicology studies are run in a highly regulated environment, and there is a relatively conservative mind-set within the pharmaceutical toxicology community. As a result, the basic design of the 1-month rodent and non-rodent toxicology studies has remained largely unchanged since the 1970s.

7 Conclusions

The kidney plays a crucial role in various physiological processes such as fluid and electrolyte balance, control of blood pressure, and excretion of endogenous and exogenous products, including drugs. Drug-induced kidney injury remains a significant cause of drug attrition during clinical development and its mechanisms are still poorly understood. Therefore, although not part of the ICH S7A core battery, preclinical assessment of potential risk of DIKI might be important for the pharmaceutical industry. Renal function is mainly investigated in vivo, in stand-alone safety pharmacology studies including assessment of glomerular and hemodynamic function or by integrating renal/urinary analyses in repeat-dose toxicity studies. In addition to the standard clinical chemistry parameters and microscopic examination

of kidney tissue, novel promising renal biomarkers have emerged, offering greater sensitivity and specificity than traditional parameters.

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Inclusion of Safety Pharmacology Endpoints in Repeat-Dose Toxicity Studies

Will S. Redfern

Contents

Abstract

Whereas pharmacological responses tend to be fairly rapid in onset and are therefore detectable after a single dose, some diminish on repeated dosing, and others increase in magnitude and therefore can be missed or underestimated in single-dose safety pharmacology studies. Safety pharmacology measurements can be incorporated into repeat-dose toxicity studies, either routinely or on an ad

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hoc basis. Drivers for this are both scientific (see above) and regulatory (e.g. ICH S6, S7, S9). There are inherent challenges in achieving this: the availability of suitable technical and scientific expertise in the test facility, unsuitable laboratory conditions, use of simultaneous (as opposed to staggered) dosing, requirement for toxicokinetic sampling, unsuitability of certain techniques (e.g. use of anaesthesia, surgical implantation, food restriction), equipment availability at close proximity and sensitivity of the methods to detect small, clinically relevant, changes. Nonetheless, 'fit-for-purpose' data can still be acquired without requiring additional animals. Examples include assessment of behaviour, sensorimotor, visual and autonomic functions, ambulatory ECG and blood pressure, echocardiography, respiratory, gastrointestinal, renal and hepatic function. This is entirely achievable if the safety pharmacology measurements are relatively unobtrusive, both with respect to the animals and to the toxicology study itself. Careful pharmacological validation of any methods used, and establishing their detection sensitivity, is vital to ensure the credibility of generated data.

Keywords
3Rs • Dog • Functional observational battery • ICH S9 • Minipig • Monkey • Mouse • Noninvasive telemetry • Rat • Safety pharmacology endpoints

1 Introduction

The primary purpose of single-dose safety pharmacology studies is to protect healthy volunteers in Phase I clinical trials from acutely harmful effects of new candidate drugs (ICH S7A; Anon. 2000; Redfern et al. 2002). Phase I clinical trials are reasonably safe (Sibille et al. 2006), partly as a result of effective preclinical testing strategies (including safety pharmacology studies) to eliminate high-risk safety liabilities. More subtle but potentially impactful toxicities often emerge only when drugs are administered for longer periods of time to large patient populations. Adverse effects can occur after acute (i.e. single dose) administration or repeated dosing and can be functional and/or structural in nature. In fact, many of the recent high-profile examples of drug-induced toxicities in humans were functional in nature or in origin (Redfern et al. 2013). This chapter aims to illustrate how the value of in vivo toxicology studies can be enhanced by including the measurement of safety pharmacology endpoints. This is an area of increasing interest (Redfern et al. 2013; Authier et al. 2013).

In addition to adding an extra dimension to the information content of toxicology studies, the inclusion of safety pharmacology endpoints would have a 3Rs benefit (Russel and Burch 1959), particularly if this could be achieved without the use of additional animals and without significantly increasing the welfare burden on individual animals involved in these studies. In particular, it would assist with reducing the numbers of animals used, firstly by minimising or obviating the need

for standalone repeat-dose investigative studies addressing a specific functional endpoint and, secondly, by providing clearer information to prevent compounds with problematic safety profiles progressing into further, extensive nonclinical in vivo regulatory toxicology evaluations, only to be stopped eventually because of adverse effects.

2 Principal Aims of Repeat-Dose Toxicity Studies

In vivo toxicology studies are conducted at any point from early drug discovery (to assess adverse effects related to the intended molecular target and/or chemical series) through to late stage development (i.e. regulatory studies to support clinical trials and marketing). The primary aim of regulatory repeat-dose toxicity studies in animals is to identify the potential hazards of new chemical entities (NCEs). This is achieved by evaluating target organ toxicities and the exposure levels at which these occur, thereby enabling detection of potential safety hazards, and safety risk assessment, before human exposure.

The standard duration of the regulatory toxicology studies run prior to human exposure is 28 days of dosing ('1-month studies'), which permits dosing of the same duration in humans (Keller and Banks 2006; Baldrick 2008; Anon 2009a; Sparrow et al. 2011); they are required in a rodent and a non-rodent species and are conducted according to the principles of Good Laboratory Practice (GLP; Anon 2009a, b). These 1-month studies are preceded by maximal tolerated dose/dose range-finding (MTD/DRF) studies. The MTD phase involves dose escalation and adjustment until the maximal tolerated dose is established for a single administration. The DRF phase involves administering the MTD (alongside lower doses and a vehicle control group), usually for 7 or 14 days. The MTD/DRF studies provide a degree of confidence that a 1-month study can proceed safely and successfully using these doses throughout (Baldrick 2008; Herlich et al 2009). Longer-term toxicology studies are conducted in parallel to early clinical trials to enable the dosing of patients over longer periods (Keller and Banks 2006; Baldrick 2008; Anon 2009a, b; Sparrow et al. 2011).

The primary endpoints of general toxicology studies are pathological ones: histopathological examination of a standard set of tissues postmortem. In addition, during the in-life phase of the study, blood samples are taken for assessment of effects of the test compound on haematology and plasma chemistry, and for measurement of plasma exposure to the test compound ('toxicokinetics'), urine is collected for urinalysis, and ophthalmoscopy is performed. The only physiological assessments made routinely are of body weight, food consumption (per cage, in the case of rodents), urine biochemistry and (in non-rodent species), electrocardiogram (ECG) (Table 1). A key outcome of the studies is the determination of a 'no-observed-adverse-effect level' (NOAEL; Dorato and Engelhart 2005).

Table 1 In-life measurements and blood sampling on main study animals during a repeat-dose toxicity study

	Rodent	Non-rodent
Toxicokinetic sampling	Usually on satellite groups; microsampling (Chapman et al. 2014) may change this, in which case usually multiple sampling on Day 1 and at end of dosing phase	Usually multiple sampling on Day 1 and at end of dosing phase
Clinical observations	Daily (?)	Daily (?)
Body weight	At least weekly	At least weekly
Food consumption	At least weekly	At least weekly
Ophthalmoscopy	Pre-study, end of dosing phase	Pre-study, end of dosing phase
ECG	Not routine	Pre-study, end of dosing phase
Haematology and clinical chemistry	At least once during dosing phase	Pre-study, at least once during dosing phase
Urinalysis	Metabolism cage, at least once during dosing phase	Metabolism cage or bladder catheterization, pre-study and at least once during dosing phase

Modified from Keller and Banks (2006)

3 Drivers for Inclusion of Safety Pharmacology Endpoints in Repeat-Dose Toxicity Studies

These are of two types: scientific and regulatory. Together, these drivers have stimulated interest in the inclusion of safety pharmacology endpoints in repeat-dose toxicity studies on conventional pharmaceuticals (i.e. non-biologics) (Redfern and Valentin 2011; Redfern et al. 2013).

3.1 Scientific Drivers

Early calls for inclusion of safety pharmacology endpoints arose from within the toxicology community, led by Gerhard Zbinden from the late 1970s onwards (Alder and Zbinden 1983; Zbinden 1984, 1991) and subsequently echoed by others (Matsuzawa et al. 1997; Luft and Bode 2002; Harkin et al. 2003; Redfern et al. 2013), although recommendations to include neurobehavioural assessments within toxicity studies date back even earlier (Ruffin 1963; Brimblecombe 1979).

Another scientific argument for evaluating safety pharmacology effects on repeat-dosing is the phenomenon of delayed-onset effects that may be missed if just assessing the effects of a single administration. Mechanisms for delayed effects

include both pharmacokinetic ones (e.g. progressive accumulation in the body or in a particular organ, especially the heart or brain), drug metabolism (formation of a pharmacologically active metabolite), and changes in cellular biochemistry (receptor downregulation; upregulation; inhibition of ion channel trafficking, e.g. hERG; inhibition of axonal transport; etc.). Although the first two mechanisms would be expected to manifest themselves within the 24 h timeframe of a single-administration safety pharmacology study, there are exceptions, including the delayed onset, progressive effect of amiodarone on QT interval due to myocardial accumulation over days and weeks (Kodama et al. 1997).

Some functional changes can *precede* the onset of pathological damage revealed by standard histopathological techniques or occur at lower exposures and can therefore provide a clinically translatable biomarker to inform and limit dose escalation in clinical trials and may also provide potential earlier endpoints in terms of animal welfare. Other functional changes are accurately *coupled to* the time course of the pathology and thereby mirror the pathological changes without the requirement for termination of animals for histopathology at different time points. Finally, some pathological changes occur *as a consequence of* functional changes, for example, specific cardiac lesions in dogs (Dogterom et al. 1992) and monkeys (Misner et al. 2012) developing as a result of sustained episodes of drug-induced tachycardia and/or hypotension.

3.2 Regulatory Drivers

In regulatory toxicology, the primary drivers for change have always been regulatory requirements, either implicit (in guidelines) or implied.

The 1997 ICH Guidance document (S6) on preclinical safety assessment of biopharmaceutical products (Anon 1997, 2011) indicated that safety pharmacology endpoints could be assessed during toxicology studies, rather than requiring stand-alone studies. FDA guidance on exploratory IND studies (i.e. clinical trials of limited exposure on NCEs for which there is no therapeutic intent) also encouraged the integration of safety pharmacology measurements into toxicology studies (Anon. 2006a), thus reducing the necessity for stand-alone safety pharmacology studies. The revised ICH Guidance document (S9) on oncology products (Anon. 2009b) also largely removed the regulatory requirement for stand-alone safety pharmacology studies where such products were destined for evaluation in patients with end-stage cancer. In addition, for logistical reasons, safety pharmacology assessments are often incorporated into inhalation toxicology studies in rodent and non-rodent species, rather than performing separate studies. These regulatory drivers have largely offered an ‘instead of’ (cost-saving) rather than an ‘as well as’ (value-adding) approach, which does give some cause for concern. Nonetheless, at least they provide opportunities for toxicologists and safety pharmacologists to work together to achieve meaningful functional observations and measurements within the constraints of repeat-dose toxicity studies.

The only regulatory driver thus far for more extensive, higher quality functional assessments in repeat-dose toxicity studies in the pharmaceutical industry has been in the area of QT prolongation and torsade de pointes (TdP, see below). Although there has been less focus on other areas of safety pharmacology, nonetheless there are growing concerns that subtle functional changes may lead to undesirable and unacceptable safety risks to patients (e.g. increased incidence of cardiovascular adverse events or elevated cardiovascular risk factors with drugs used to treat coronary heart disease, diabetes, obesity, arthritis, attention-deficit hyperactivity disorder and psychiatric disorders) (Barter et al. 2007; Kaul and Diamond 2010; Aw et al. 2005; Scheen 2010; Scheiman and Hindley 2010; Santosh et al. 2011; Mehta et al. 2011). Such liabilities only emerged after chronic therapy, which therefore suggests missed opportunities to be addressed in repeat-dose toxicology studies.

In addition, certain specialised regulatory requirements include the incorporation of specific behavioural measurements. These include regulatory guidelines on postnatal development, requiring behavioural testing of first-generation offspring exposed to NCEs during gestation (Anon. 2005, 2008), and juvenile toxicity studies, where neonatal and/or juvenile animals are dosed and assessed using behavioural tests (Anon. 2006b, 2008; Baldrick 2010). The behavioural tests include a functional observational battery (FOB), locomotor activity, motor coordination (e.g. rotarod) and learning and memory tests. So if such behavioural assessments are incorporated in these specialised studies, then in principle, they could be included in general toxicity studies.

4 Challenges and Opportunities

The primary aim of a repeat-dose toxicity study is to expose animals to different levels of a test compound over a prolonged period, assess in-life indicators of systemic toxicity and, finally, evaluate histological changes postmortem (Table 1). Any additional safety pharmacology measurements should not and must not interfere with these aims or affect their outcome. However, the study design, laboratory design and environment may not be optimal in terms of obtaining high-quality safety pharmacology data. Compare the custom-designed, fit-for-purpose rodent safety pharmacology suite design of Fig. 1a to a typical rodent toxicology study holding (and ante) room in Fig. 1b. Comparing toxicology and safety pharmacology studies gives some insight into potential operational differences (Table 2). Achieving integration of meaningful safety pharmacology assessments into toxicology studies requires certain compromises and adjustments. For example, depending on the study type and functional measurements, it may require a staggered start date (to enable measurements across all the animals in the main study group, e.g. spread over two consecutive days), staggered dosing on the day of safety pharmacology assessments (to enable measurements at the T_{max} time point) and so on.

An important scientific issue is the phenomenon of tolerance to the effects of repeat-dosing. This can occur with many classes of drug and in various organ systems. This poses something of a problem. The first day of dosing (Day 1) of a repeat-dose toxicity study is generally the busiest one for the staff; it is already fully

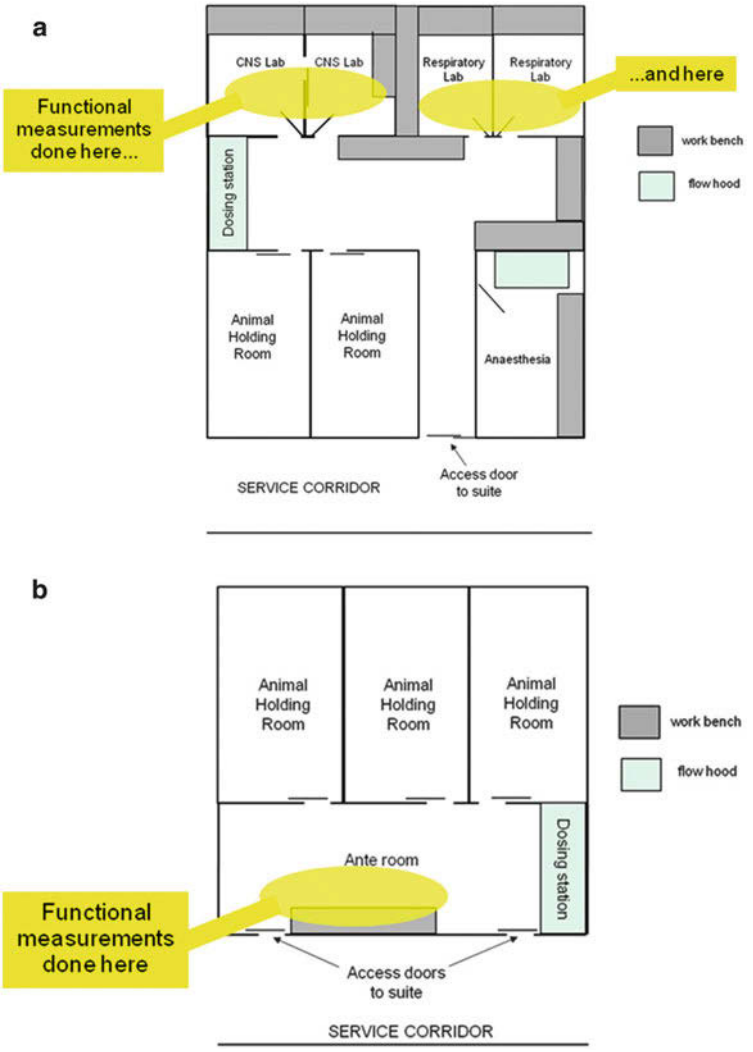

Fig. 1 (**a**) *Example of a custom-designed, fit-for-purpose rodent safety pharmacology suite*. Features: testing labs located remote from corridor noise (e.g. trundling of cage racks, loud conversations). Primary access to suite via single entry door, with warning to limit entry to essential visits and to minimise noise level. Staff requiring access to the other animals on the study can do so without disturbing the safety pharmacology observations/measurements. Entry to the testing labs restricted to staff involved in the observations/measurements. Designed to accommodate bulky test equipment, ergonomically. Lighting control with local (manual) override. Similar principles would apply to large animal cardiovascular measurements, with as monitoring suite remote from (rather than adjacent to) the animal accommodation. (**b**) *Example of typical rodent toxicology study holding rooms with ante room.* Drawbacks: testing area adjacent to corridor noise (e.g. trundling of cage racks, loud conversations). Access from corridor directly into testing area. Staff requiring access to the other animals on the study disturb the safety pharmacology observations/measurements. Entry to the testing area unrestricted. Bulky test equipment may be difficult to accommodate ergonomically. Automated lighting control with no manual override. Similar drawbacks occur in large animal toxicology facilities, often with data monitoring in an adjacent room (with human entry/activity/conversations detected by the animals)

Table 2 Differences in emphasis and operational paradigms between general toxicology and safety pharmacology studies

	General toxicology	Safety pharmacology
GLP	Yes, inviolable for regulatory studies; not required for DRF/MTD studies	Yes (for core battery studies), although some software may not be fully GLP-compliant; also, noncore battery studies need only to be conducted to GLP 'to the greatest extent feasible'
Primary adverse effect type predicted	Types C–E (B)	Type A
Primary endpoints	Gross clinical signs; histopathology. Studies scheduled to accommodate necropsy slots	Functional responses/effects. No necropsy to consider
Dosing regimen	Chronic, repeat-dose; animals dosed all in one session (usually a.m.)	Single dose (usually); dosing staggered to accommodate functional measurements
Dose or exposure level	Lowest dose tested can be much larger than the anticipated 'therapeutic' dose. Highest dose usually the MTD or maximum dose of (usually) 1 g/kg	Lowest dose tested is around the 'therapeutic' dose level; highest dose may be the single-dose MTD
Cardinal exposure parameter	AUC; TK sampling takes priority	C_{max} TK sample taken *after* key functional measurements
Sex of animals	Males and females	Usually males
Age of animals	Sexually mature animals used	Sexually mature non-rodents used, but behavioural studies usually require young rats
Strain of animals	Usually restricted to standard strains	May sometimes require non-standard strains (e.g. pigmented rats)
Training/ habituation	Variable	Some functional measurements may require pre-training and/or habituation of animals
Facilities	Often busy, noisy environments. May be difficult to accommodate bulky equipment or to adjust lighting levels, etc.	Functional measurements require a quiet room; usually conducted in custom-designed facilities with lighting controls, etc.
Basis for risk assessment	NOEL, NOAEL	Margins
Statistical analysis	Trend tests; group sizes adequate to detect histopathological effects	Within-animal pairwise comparisons of means or ANOVA compared to vehicle controls; studies powered to detect the functional effect
Study design	Established discipline	Continuously evolving discipline
Staff education/ expertise	Toxicology; biochemistry; molecular biology; veterinary	Pharmacology; physiology

loaded with repeated toxicokinetic blood sampling at specific time points, and the procedure rooms are relatively crowded with the toxicology technicians, study director, quality assurance auditor (in GLP studies) and even the sponsor's monitoring scientist (in the case of a study run at a CRO). This is really a day to avoid for quiet and careful measurement of physiological and behavioural parameters. But to opt for (say) Day 2 instead may miss the peak effect of first administration, which may have faded or changed entirely on second administration. This is not an issue when functional measurements in repeat-dose toxicity studies are 'in addition to' single-dose safety pharmacology studies, but it could be when they are run 'instead of'.

5 Criteria for Suitable Methodology

Methods to assess safety pharmacology endpoints in repeat-dose toxicity studies should ideally be noninvasive, non-stressful, not require anaesthesia and not require food restriction. Otherwise, satellite groups should be considered or even a separate study performed. Equipment needs to be portable and relatively non-bulky: ideally, the equipment should be moved to where the study animals are housed rather than the other way round. Alternatively the equipment can be an integral part of the facilities and purposely dedicated to measurements (e.g. noninvasive telemetry receivers). Measurements should be conducted by scientists and technicians experienced in how to acquire high-quality safety pharmacology data and in collecting, analysing and interpreting it. This is easier to achieve if safety pharmacology and general toxicology departments are in close proximity, with the safety pharmacology staff incorporated within the GLP Compliance Programme. The design of the study should be discussed with a statistician ahead of the study where possible, particularly with respect to statistical power and any requirement for pre-study measurements. Toxicology studies may be underpowered relative to stand-alone safety pharmacology studies to detect some functional changes and are likely to include equal numbers of males and females. For example, sensitivity of detection of changes in QT interval using conventional (manual) ECG in toxicology studies in dogs is lower than in telemetered dog safety pharmacology studies (Hammond et al. 2001; Tattersall et al. 2006; Guth et al. 2009). However, the introduction of noninvasive telemetry systems has enabled a significant improvement in the sensitivity of toxicology studies in relation to detection of QTc changes. Ideally, data recordings should be at the same time(s) post-dose for each animal, at roughly the same time of day throughout the study, and should (where technically possible) collect data over a 24 h period. Techniques used for long-term monitoring need to be suitable for use in group-housed (or pair-housed) animals; certainly in the EU, social housing will become a welfare requirement (Anon. 2010). Finally, apart from certain noncompliant data acquisition software systems, there is no reason why GLP compliance cannot be claimed for this aspect of the study.

Examples of suitable methods for application to main study animals in repeat-dose toxicity studies are given in Table 3.

Table 3 Examples of safety pharmacology assessments suitable for incorporation into repeat-dose toxicology studies

Organ system/function	Technique/method	Species
Cardiovascular system		
ECG interval durations, amplitude and morphology; heart rate	Conventional 'snapshot' recordings in restrained animals	Dog; monkey; minipig
ECG interval durations, amplitude and morphology; heart rate	Surface electrodes using noninvasive telemetry in freely moving animals	Dog; monkey; minipig
Left ventricular function	Echocardiography	Dog; rat
Arterial blood pressure; heart rate	Tail cuff (restrained)	Dog; rat
Arterial blood pressure; heart rate	Ambulatory tail cuff (telemetry)	Dog
Arterial blood pressure; heart rate	Minimally invasive blood pressure	Dog; monkey
Nervous system		
Global neurobehavioural assessment	Functional observational battery or Irwin test	Rat; mouse; dog; monkey
Ambulatory activity (home cage)	RFID microchip transponder	Rat; mouse
Ambulatory activity (novel arena)	Locomotor activity (photocell beam breaks or video tracking)	Rat; mouse
Motor coordination	Accelerating rotarod; beam walking; gait analysis	Rat; mouse
Cognitive function	Avoidance paradigms (brief footshock stimulus on a single occasion)	Rat; mouse
Auditory function	Brainstem auditory evoked response	Dog
Auditory function	Pre-pulse modulation of startle reflex; startle stimulus-response curves	Rat; mouse
Visual acuity	Optomotor reflex	Rat; mouse
Iris control	Pupil diameter ; pupillary reflex response to light stimulus	Rat; dog
Nociception	Tail flick latency	Rat; mouse
Neuromuscular	Grip strength	Rat
Salivation	Absorption into preweighed gauze	Dog
Respiratory system		
Respiration rate, inspiratory and expiratory times, tidal volume, minute volume, peak inspiratory and expiratory flows	Whole-body plethysmography	Rat; mouse
(Ditto)	Inductive plethysmography	Dog; monkey
Renal system		
Water intake, urine volume, urinary excretion of key electrolytes (Na^+, K^+, Cl^-). Estimated GFR and fractional excretion of electrolytes	Urine collection in metabolic cages	Rat

(continued)

Table 3 (continued)

Organ system/function	Technique/method	Species
Gastrointestinal system		
General assessment	Faeces weight, consistency, appearance	Rat, Dog
Gastric emptying time; intestinal transit time; intestinal pressures	Telemetry capsule	Dog
General metabolic functions		
Rectal temperature	Rectal temperature probe (thermocouple or thermistor)	Rat; mouse; dog
Subcutaneous temperature (e.g. interscapular temperature)	RFID microchip transponder	Rat; mouse
Glycaemic control	Glucose tolerance test (serial blood microsampling)	Rat; mouse; dog; monkey; minipig
Mitochondrial function	Blood glucose and lactate	Rat; mouse; dog; monkey; minipig
Oxygen consumption	Whole-body indirect calorimetry	Rat; mouse

For source references for each of these techniques across different species used in toxicology studies, refer to text and to Redfern et al. (2013)

6 Application to Specific Organ Functions

The five organ functions assessed in safety pharmacology are the cardiovascular system, nervous system, respiratory system, gastrointestinal system and renal system. To these we would add general systemic metabolic functions, as these are amenable to collection of at least some biochemical and physiological data.

6.1 Cardiovascular System

6.1.1 ECG Intervals and Morphology

In recent years, jacketed, noninvasive, ambulatory telemetry systems have become commercially available for use in dogs and primates (Chui et al. 2009; Prior et al. 2009a; Ward et al. 2012; Soloviev et al. 2007). These enable continuous recordings of lead II ECG from surface electrodes for at least 24 h in undisturbed, freely moving animals in their home environment, when awake or asleep. The same requirements for on-the-spot cardiovascular expertise apply as for conventional ECG recordings. In addition, a detailed knowledge and understanding of the daily routines and activities within the animal housing unit (e.g. feeding times, presence of technicians, husbandry activities, etc.) is required in order to design appropriate studies and make intelligent interpretations of the data. Access to the housing area

(and adjacent rooms) should be restricted and all entries logged. Data quality obtained from jacketed telemetry in beagle dogs is superior to that obtained conventionally using 'snapshot' recordings, including achieving lower resting heart rates (Redfern et al. 2013), and there is more of it. This approach enables determination of onset, time course, duration, magnitude and reversibility of effects as well as dose-dependence and PK-PD relationships. Plots of QT against RR interval circumvent the inaccuracies associated with QT correction factors and can reveal drug-induced shifts across the spectrum of heart rates (Markert et al. 2004).

In contrast to detecting effects on the QT interval, sampling during naturally occurring episodes of elevated heart rates facilitates detection of drug-induced QRS prolongation by sodium channel blockers (Cros et al. 2012), as in contrast to the hERG potassium channel, sodium channel block is use-dependent (i.e. rate-dependent) (Weirich and Antoni 1998). Detection of cardiac arrhythmias is more likely over a 24 h sampling period than with snapshot ECGs, as is the detection of pre-existing waveform abnormalities pre-study (Macallum and Houston 1993; Cools et al. 2011; Leishman et al. 2012). The impact on resources of using a jacketed telemetry system is minimal (Redfern et al. 2013), although an initial significant capital investment is required. When used to complement stand-alone, single-dose safety pharmacology studies in dogs with surgically implanted telemetry devices, the noninvasive ECG measurements can sometimes reveal effects that intensify on repeated dosing.

6.1.2 Left Ventricular Function

The use of echocardiography in unanaesthetised dogs is a well-established technique (e.g. Crosby et al. 1984). Left ventricular function can be monitored using echocardiography in unsedated, pretrained, sling-restrained (or laterally recumbent) dogs (Hanton et al. 2004, 2005; Prior et al. 2009b; Cooper et al. 2011) and minipigs (Konrad et al. 2000). Echocardiography can also be used in cynomolgus monkeys, but they require sedation (Sleeper et al. 2008) or anaesthesia (Tsusaki et al. 2005). In beagle dogs, good concordance is observed between left ventricular ejection fraction (LVEF) derived by echocardiography and left ventricular dP/dt_{max} measured in the same animals by implanted telemetry (Prior et al. 2009a; Cooper et al. 2011). The QA interval (the time between the Q-wave of the ECG and upstroke of the aortic pressure wave) can also be used as an index of ventricular contractility (Norton et al. 2009). This would require simultaneous recording of ECG and the arterial blood pressure waveform (using minimally invasive blood pressure recordings; see below).

6.1.3 Arterial Blood Pressure

In rodents, indirect (i.e. noninvasive) methods of blood pressure measurement utilise a tail-cuff method. However, they can only reliably measure systolic blood pressure and heart rate and require prewarming and restraint to obtain the measurements, and even after pre-study habituation to these recording conditions, the values of systolic blood pressure and heart rate obtained are elevated compared

to unrestrained animals with implanted telemetry devices (Kurtz et al. 2005; Authier et al. 2015). Recent improvements in tail-cuff blood pressure methodology are a promising development (Feng et al. 2008), but even so, such a method requires highly skilled operators and a dedicated, quiet environment. Given that regulatory repeat-dose toxicity studies are carried out in two species, it would seem sensible to run cardiovascular assessments in the larger, non-rodent species, although cardiovascular measurements in rodents may be informative in non-regulatory, DRF studies, especially given the low group sizes used in non-rodent DRF studies. The tail-cuff blood pressure method itself is also well established in dogs (Haberman et al. 2006; Mitchell et al. 2010), and the recent introduction of high-definition oscillometry (HDO) enables capture of the blood pressure waveform, with accurate measurements of systolic, diastolic and mean arterial blood pressures (Meyer et al. 2010; Sarazan 2014; Egner 2015). HDO has also been used in cynomolgus monkeys (Schmelting 2009, 2011; Schmelting et al. 2009, 2010; Mitchell et al. 2010; Sarazan 2014).

Ambulatory tail-cuff methods are available for dogs, combining simultaneous ECG and blood pressure measurements in the same animals by noninvasive telemetry (Schmelting 2011; Bailey et al. 2012; Ward et al. 2012). An alternative approach involves a minimally invasive measurement of blood pressure in dogs or cynomolgus monkeys via an arterial catheter connected to a miniature (rodent) telemetry transmitter. Signals are recorded either with the dog in a sling, or by using a signal booster housed in a collar, or in a jacket (e.g. ECG external telemetry jacket) (McMahon et al. 2010; Niehoff et al. 2014) to enable continuous, high-quality 24 h recordings in freely moving animals. It is preferable to position the catheter tip at approximately the same location as would be done using conventional telemetry to avoid differences in blood pressure readings between the two techniques due to changes in waveform morphology (Sarazan 2014).

6.2 Nervous System

Standard approaches to detect drug-induced neurotoxicity (functional and pathological) are limited to a single-dose neurobehavioural assessment as part of the safety pharmacology core battery and 3–4 coronal sections through the entire neuraxis for histopathology. Reliable blood-borne biomarkers of drug-induced neurotoxicity do not currently exist. This is therefore an area of relative neglect in preclinical toxicology evaluation, and so functional assessments in repeat-dose toxicity studies are worth considering. Technically, there is nothing preventing routine inclusion of global neurobehavioural assessments (by a trained observer) and automated activity monitoring in repeat-dose toxicity studies, and evaluation of specific neurobehavioural systems could be incorporated on a case-by-case basis.

6.2.1 Global Neurobehavioural Assessment

Due to the extreme diversity and complexity of nervous system functions, it is not possible to devise a convenient in vivo assessment that incorporates all of them.

Therefore, in safety pharmacology, the first-tier test used is a neurobehavioural assessment, either in the format of the functional observational battery (FOB) or Irwin test (Moser 1989; LeBel and Foss 1996; Moser et al. 1997; Redfern et al. 2005; Roux et al. 2005; Redfern and Wakefield 2006). Similar assessments have been devised for dogs (Gad and Gad 2003; Tontodonati et al. 2007; Moscardo et al. 2009) and monkeys (Gauvin and Baird 2008; Moscardo et al. 2010; Froget et al. 2012). Where there is specific cause for concern arising from the knowledge of a compound, appropriate single-parameter tests can be incorporated into a repeat-dose toxicity study on a case-by-case basis to address the issue. Examples of suitable tests are listed in Table 3.

6.2.2 Automated Activity Analysis

Activity of rodents can be assessed in two ways: home cage activity or activity in a novel enclosure (spontaneous locomotor activity). These are quite different measures. The latter involves a conflict between the motivation to explore and raised anxiety in unfamiliar surroundings. It can be monitored using either arrays of photocell beams (with a second set positioned to detect rearing) or by video tracking (Vorhees et al. 1992; Patterson et al. 2005; Himmel 2008; Lynch et al. 2011). Ambulatory activity and rearing in a novel environment is initially high and gradually decays over 30 min or so, as the animal explores and habituates to its surroundings, eventually spending the majority of time either stationary or grooming (Redfern and Wakefield 2006). Subsequent exposure to the same (or similar) test arena results in habituation, with reduced activity after the first trial or increased data variability (Golozoubova et al. 2014), impacting on its suitability for longitudinal testing in a repeat-dose toxicity study. Home cage activity does not have the conflict or habituation components, providing a reproducible 24 h cycle of activity for each individual. Both types of activity are affected by time of day, light-dark cycle, extraneous noise and the presence of human observers. A sedative effect of a test compound would be difficult to detect on home cage activity of rodents during the light phase, as this is already very low (Leslie et al. 2014). Nonetheless, in a repeat-dose toxicity study, the most convenient format to use would be home cage activity. Until fairly recently, this has required single-housing of animals (Smits et al. 2008) or surgical implantation of telemetry devices (Vinkers et al. 2009), neither of which is ideal for a repeat-dose toxicity study.

Technology is emerging for measuring activity, behaviours and temperature of individual rodents when group-housed in their home cage, without requiring surgical implantation of telemetry transmitters. Techniques exist for measuring individual ambulatory activity in group-housed rodents in their home cage, using a radiofrequency identity microchip transponder ('RFID chip') implanted by subcutaneous injection, detected by a base plate reader placed under the cage (Onishchenko et al. 2011); this approach can also combine measurements of activity and temperature (Leslie et al., 2015). It is also possible to monitor individual food and water consumption in group-housed rodents in the same way (Krohn et al. 2010). Such methodology could also be useful in detecting changes in activity,

behaviour and body temperature occurring during withdrawal from treatment with drugs carrying a physical dependence liability (Froger-Colléaux et al. 2011), if applied to the recovery groups in a 1-month rat study.

Activity of dogs (Yam et al. 2011) and monkeys (Mann et al. 2004; Papailiou et al. 2008) can be conveniently measured in their home enclosures using an accelerometer placed in a collar or as an integral part of a noninvasive telemetry system. Quantitative video tracking of group-housed cynomolgus monkeys can be achieved using differently coloured jackets (Rose et al. 2012).

6.2.3 Tests on Specific Neurobehavioural Systems

Examples of 'single-parameter tests' that can be incorporated into repeat-dose toxicity studies include evaluation of the risk of (a) visual dysfunction, assessed using an optomotor method in rodents (Redfern et al. 2011a); (b) mydriasis and impaired pupillary light reflex, assessed by measuring pupil diameter in rodents and dogs (Redfern et al. 2007); (c) increased or decreased salivation in dogs, measured using a preweighed gauze swab placed in the jowl (Redfern et al. 2013); (d) impaired muscle function, assessed by accelerating rotarod performance in rats (Nodop Mazurek et al. 2011; Redfern et al. 2013); and (e) peripheral neuropathy, assessed by the same method in rats or using a beam walking task (Stanley et al. 2005). Such assessments can significantly contribute to decision-making in projects, either in terms of compound progression, design of Phase I clinical trials or development of risk management and mitigation plans.

6.3 Respiratory System

Respiratory parameters can easily be acquired from rodents (usually rats) using whole-body plethysmography, a noninvasive, non-restraint method that measures small changes in chamber pressure as a consequence of inspiration and expiration (Murphy 2002; Stonerook 2015). Stand-alone safety pharmacology studies using whole-body plethysmography typically involve a 60–90 min settling period prior to compound administration to ensure a steady respiratory signal and to reduce any artefacts in the signal due to ambulatory movement, sniffing, grooming or rearing behaviours. Post-dose recording usually continues for up to 6 h. The technique must employ appropriate temperature and humidity compensation to ensure an accurate determination of tidal volume (Jacky 1980). Application of this technique into repeat-dose studies is feasible (Ewart et al. 2008) but, depending on the number of chambers recorded from simultaneously, will generally require a staggered start date for the study to accommodate 4 h recordings from 32 rats (vehicle plus three dose levels) run over (say) 2 days. For early toxicology studies (e.g. DRF) a condensed protocol can be used, with recording around the T_{max} only and comparing just the high-dose and vehicle control group, enabling detection of potential respiratory 'flags' for a particular compound with all the animals tested within a single 2–3 h session. For this, rats are placed in the chambers for 1 h, with the first 45 min as habituation and the final 15 min for recordings, timed to be around the

T_{max} (Redfern et al. 2011b). For inhalation toxicology studies, simultaneous measurement of respiratory parameters can be achieved using head-out plethysmography (Nirogi et al. 2012).

Recent technological advances enable measurement of respiratory parameters in dogs using a non-restraint, jacketed telemetry method. This technique is known as respiratory inductive plethysmography (Neumann et al. 1998) and involves the placement of flexible belts around the thorax and abdomen, enabling continuous measurement of respiratory parameters for up to 24 h (Murphy et al. 2010). This noninvasive telemetry technique also enables simultaneous measurement of cardiovascular parameters (Bailey et al. 2012) and can also be adapted to nonhuman primates (Soloviev et al. 2007; Kearney et al. 2011) making it an attractive proposition for inclusion in repeat-dose toxicity studies.

6.4 Gastrointestinal System

The majority of drug candidates are administered orally, and therefore the gastrointestinal tract (particularly the stomach) is exposed to a far higher concentration of drug than any other organ system. As a result of this, the incidence of gastrointestinal side effects is relatively high throughout clinical development and postmarketing (Redfern et al. 2010).

Useful data can be obtained inexpensively simply by analysing the number and appearance of faecal pellets in rodents. This is ideally best performed when animals are singly housed in metabolic cages (as is usual practice during a repeat-dose investigation to assess renal function) or in whole-body plethysmography chambers (Marks et al. 2013). Though not a quantitative approach, large changes in gastrointestinal function should be detectable, which would be valuable in an early toxicology study (e.g. DRF study).

A simple, nonlethal, charcoal meal method, which measures the rate of gastrointestinal transit, has been described for use in mice and is suitable for inclusion in toxicology studies (Marona and Lucchesi 2004). Food is withdrawn 3 h pre-dose, and mice are dosed with test compound. An aqueous suspension of charcoal is administered by oral gavage, and food is restored 1 h later. Animals are observed at 5 min intervals until the first appearance of charcoal-containing faeces, which is taken as the gastrointestinal transit time. Potential issues with this method include the variability of the data and adsorption of some drugs onto the charcoal. An elegant method has been described in rats using a small (0.85×1.5 mm), coated, magnetic dipole administered by oral gavage. The rat is then placed in a restraining tube on a sensor matrix which tracks the progression of the magnet through the gastrointestinal tract during a series of 20 min recordings (Guignet et al. 2006).

Dogs have been used extensively as an animal model of gastric and intestinal function in humans in the published literature, but to a much lesser extent in toxicology. The nonsurgical methods used have included gamma scintigraphy, radiographic imaging using radio-opaque material, tracer studies using breath analysis, administration of a telemetric capsule and pharmacokinetic measurement

of drug markers in plasma (Martinez and Papich 2009; Sjödin et al. 2011). Telemetric capsules, marketed for human use, have been used in dogs to assess adverse effects on gastrointestinal function (Penton and Norton 2009; Boillat et al. 2010a, b; Sagawa et al. 2009). These are small transmitters which are dosed orally and measure temperature, pressure and pH. Gastric emptying is detected by the sudden increase in pH when exiting the stomach into the duodenum. Measurement of intestinal transit requires the measurement of temperature or pressure to detect the time of excretion in the faeces. A current problem with capsule methods in the dog is that the relatively large capsule size prolongs the time it takes to exit the stomach into the duodenum, thereby reporting abnormally long gastric emptying times; another, more practical issue is re-ingestion by the dog (or by another dog).

Other approaches are suitable only for specific components of gastrointestinal function. For the gastric emptying component per se, ultrasonography can be used in unsedated, gently restrained beagle dogs. This merely requires measurements of the amplitude and frequency of antral contractions, collected in 3 min samples at 30 min intervals (Tsukamoto et al. 2011). For measuring gastric pH, samples of stomach fluid can be aspirated using a catheter within (say) a gavage tube in dogs and the pH measured with a pH metre (Akimoto et al. 2000).

6.5 Renal System

The two key functional measurements relating to the kidney are general excretory functions and glomerular filtration rate (GFR).

6.5.1 Estimates of General Excretory Function

A number of parameters that are currently included in most repeat-dose toxicity studies can give an indication of the general excretory function of the kidneys. These include urea and creatinine in the plasma and the measurement of specific gravity or osmolarity of the urine. Cystatin C has been proposed as an additional plasma marker for renal function, and it has gained traction in human medicine (Schöttker et al. 2012) and in nonclinical species (Almy et al. 2002). In urine, quantitative measures of urinary glucose and total protein (often normalised to creatinine concentration) can give an indication of the reabsorbative function of the renal tubule. The rationale for the glucose measurement is that over 99 % of filtered glucose is reabsorbed by the proximal tubule; although this process is saturable (by hyperglycaemia), if glycosuria occurs during normoglycaemia, this may indicate functional impairment in the proximal tubule (Hart 2005).

Additionally, other analytes such as the electrolytes, and pH, can be measured in urine with a view to detect perturbations. However, interpretation of any changes remains problematic with no reliable association between functional observations and underlying pathologies in most instances. In most cases, alterations in excretion can be viewed as a normal response to maintain internal homeostasis.

6.5.2 Glomerular Filtration Rate

A long-standing and broadly accepted measure of general renal function is the measurement of GFR, the rate at which fluid is filtered by the kidneys. GFR (mL/min) can be calculated using a wide number of techniques that employ variants of the equation GFR = (urine concentration × urine flow)/plasma concentration.

GFR in humans is estimated by measuring the plasma clearance of an administered marker compound (e.g. inulin) that is excreted exclusively by the kidneys and does not undergo further tubular reabsorption or secretion after glomerular filtration (Hart 2005). Intravenously administered clearance markers have been used in dogs (e.g. iohexol, Laroute et al. 1999) and rats (e.g. inulin, Gabel et al. 1996; iodixanol, Katayama et al. 2010). A noninvasive transcutaneous device has recently been developed to measure the renal clearance of FITC-sinistrin in conscious rodents (Schreiber et al. 2012). However, the use of renal clearance markers in regulatory toxicology studies could be challenged on the grounds of hypothetical concerns around potential innate toxicity or drug-drug interactions.

Alternatively, in toxicology studies, GFR can be estimated by measuring creatinine clearance rate, which is the volume of blood plasma that is cleared of creatinine per unit time (Hart 2005). Creatinine is produced naturally by muscle, and concentrations in blood remain broadly at steady state. It is freely filtered by the glomerulus, but because of additional active secretion by the peritubular capillaries, creatinine clearance slightly overestimates actual GFR.

It should also be noted that any changes in renal function may often be only temporary and reversible, with no long-term consequence for the animal. Not only does the kidney have a high functional reserve (approximately 75 %), it is capable of profound regeneration and recovery as well as marked upregulation of the function of individual nephrons.

6.6 Body Temperature; General Metabolic Functions

One physiological variable of fundamental importance is body temperature. This is not measured in toxicology studies, but may be inferred, correctly or incorrectly, by clinical observations such as ‘piloerection’ and ‘cold extremities’. Approximately 26 % of non-CNS-targeted test compounds were found to cause a small (~1 °C) decrease in rectal temperature in the rat in safety pharmacology studies at the MTD dose level (Redfern et al. 2005), which is thought to be an adaptive response to ingestion of a toxic agent (reducing cellular toxicity by reducing body temperature; Gordon et al. 2008; Gordon 2010). However, larger decreases in temperature may reflect either a more profound toxicity, a specific pharmacological effect on central thermoregulatory control, impaired metabolic heat production, vasodilatory heat loss or a physiological response to hypoglycaemia (Buchanan et al. 1991) or hypoxia (anapyrexia; Steiner and Branco 2004). In addition, mice (unlike rats) undergo bouts of torpor when food is unavailable (e.g. during protocol-imposed overnight fasts), whereby they enter a hypometabolic state and a lowered core temperature (Swoap 2008). This can also be driven by pharmacological and

toxicological mechanisms (Swoap 2008). Conversely, increases in temperature in rodent or non-rodent species may reveal other toxicological mechanisms, including skeletal muscle toxicity or an immunological response (Rusyniak and Sprague 2005). Collection of temperature data in repeat-dose toxicity studies is therefore extremely informative.

The use of a rectal thermocouple carries a potential risk of physical damage to the rectal wall, transfer of any gastrointestinal infections between individuals and contamination of vehicle control animals with traces of test compound from treated animals. In addition, core temperature increases as a result of handling (Gordon 1990), and rectal temperature underestimates true core temperature, for which measurement from the colon is required in the rat (i.e. 6–8 cm past the anal sphincter; Gordon 1990).

A completely noninvasive approach is achievable using infrared thermography. Modern thermal imaging cameras offer dual visual and thermal image capture, with fine image resolution, and can capture accurate regional surface temperatures from freely moving rodents, such as the temperature gradient from base to tip of the tail (Prior et al. 2012b; Luong and Carrive 2012). However, to measure core temperature, the angle of view has to be aligned with the axis of the ear canal (which is equivalent to core temperature at normal laboratory housing temperatures), and skin temperature measurement requires shaving (and reshaving) of fur. This might be required, for example, to reveal the skin overlying the interscapular brown adipose tissue (iBAT), a major source of adaptive non-shivering thermogenesis in rodents (Gordon 1990). Thermal imaging is invaluable for certain bespoke, problem-solving studies, but it requires a skilled operator, and data extraction is relatively time-consuming, so it is less suited for routine use.

As mentioned previously, RFID transponders exist which measure temperature and are small enough to be injected subcutaneously in rats and mice (Kort et al. 1998; Prior et al. 2012a, b). Temperature is measured with a hand-held wand receiver from freely moving animals, without contact (e.g. through the wall of the home cage). When injected subcutaneously into the interscapular region, the RFID chip is measuring temperature above the iBAT region. At normal laboratory housing temperatures, this temperature is slightly higher than rectal temperature in the rat (Prior et al. 2012a), rectal temperature being slightly lower than core temperature in this species (Gordon 1990). Drug-induced changes in thermoregulation generally result in the iBAT temperature mirroring changes in rectal temperature (Prior et al. 2012b). Although some drugs will lower core temperature by vasodilatory heat loss, with the iBAT temperature being maintained and therefore not reporting hypothermia, for most purposes in toxicology, the iBAT temperature does reflect what is happening to core temperature. The RFID chips can be sterilised and reused and are therefore relatively inexpensive. Subcutaneous temperature can also be measured via an RFID chip over 24 h (or longer) using a home cage monitoring system (Leslie et al. 2014). Whichever method of body temperature measurement is used, it is crucial to record and report ambient temperature in the housing room/procedure room.

One of many causes of hypothermia is mitochondrial toxicity, which can lead to metabolic acidosis. Benchtop analytical techniques are improving all the time and requiring smaller blood volumes (i.e. microsampling). For example, these can measure glucose, metabolic intermediates, electrolytes, blood gases and pH and have been used in rats (Keiver et al. 2005) as well as larger species (Borron et al. 2006; Eddleston et al. 2012). Assessment of glycaemic control per se is generally done using an oral or intravenous glucose tolerance test, which has been used across the toxicology species (Ishida et al. 1997; Tsunoda et al. 2008; Christoffersen et al. 2009; McGuinness et al. 2009; Wagner et al. 2010; Zong et al. 2011). Another useful measurement, when there is cause for concern, is oxygen consumption using whole-body indirect calorimetry, which reflects overall metabolic rate (Even et al. 1994). This can be measured by placing rodents in small chambers for, say, 3 h (1 h habituation, 2 h' recording; Rodríguez et al. 2002).

7 Options for Routine and Ad Hoc Inclusion of Safety Pharmacology Endpoints in Repeat-Dose Toxicity Studies

Where safety pharmacology measurements are included in repeat-dose toxicity studies *in place of* stand-alone safety pharmacology studies, we may have to attempt to conduct the measurements on Day 2 of dosing and on at least one other occasion. In doing so, we are conceding that any adverse effects detected may have been of a larger magnitude on Day 1, but that this is a trade-off to avoid adding to the complexity of Day 1. In addition, on the days of safety pharmacology measurements, the dosing should be staggered to enable the safety pharmacology assessments to be made at approximately the same time point(s) post-dose in all the animals tested. Finally, the study start-finish dates may need to be staggered to accommodate the safety pharmacology assessments on all the animals to be tested on Day 2 of dosing (and on the same study days for subsequent recordings). The logistical problems involved in doing this, particularly in trying to accommodate FOB/Irwin assessments and plethysmography recordings into a single rodent toxicology study, should not be underestimated.

Where safety pharmacology measurements are included in repeat-dose toxicity studies *in addition to* stand-alone safety pharmacology studies, more flexibility can be afforded to the measurements, thereby minimising alterations to the conventional toxicology study design. The safety pharmacology measurements should therefore be designed to have minimum impact on the conventional repeat-dose toxicity study protocol and minimum impact on the study animals. In early non-GLP toxicity evaluations (e.g. the MTD/DRF studies), where the objective of any safety pharmacology assessments is 'hazard detection' rather than 'risk assessment', we can afford to scale-down the measurements from the standard designs in safety pharmacology studies and also perhaps just assess the high-dose group and the vehicle controls. This would be sufficient to detect large effects, providing an early warning of significant effects ahead of the regulatory GLP toxicology/safety pharmacology studies, thereby enabling sufficient time for proper evaluation in an

interim, bespoke study or in the 1-month studies. In either scenario, multiple measurements can be conducted on the same animals, either on the same or different days within the study.

8 Summary and Conclusions

An augmentation of long-term toxicity studies by including safety pharmacology assessments would enable longitudinal investigation of adverse effects of candidate drugs prior to investment in the expensive phases of clinical development (Phases II and III). The option to assess safety pharmacology (i.e. functional) endpoints in repeat-dose toxicity studies in certain circumstances (e.g. biologics, terminal cancer agents, exploratory INDs) has encouraged toxicology departments to incorporate physiological and behavioural measurements into the regulatory toxicology studies (Redfern et al. 2013; Authier et al. 2013). If this can be done for these special cases, then in principle it could be done for all drug discovery projects, either routinely or on a case-by-case basis.

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Part IV

Safety Pharmacology of Biological and Anticancer Pharmaceuticals

Safety Pharmacology Evaluation of Biopharmaceuticals

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Contents

Abstract

Biotechnology-derived pharmaceuticals or biopharmaceuticals (BPs) are molecules such as monoclonal antibodies, soluble/decoy receptors, hormones, enzymes, cytokines, and growth factors that are produced in various biological expression systems and are used to diagnose, treat, or prevent various diseases. Safety pharmacology (SP) assessment of BPs has evolved since the approval of the first BP (recombinant human insulin) in 1982. This evolution is ongoing and

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is informed by various international harmonization guidelines. Based on these guidelines, the potential undesirable effect of every drug candidate (small molecule or BP) on the cardiovascular, central nervous, and respiratory systems, referred to as the "core battery," should be assessed prior to first-in-human administration. However, SP assessment of BPs poses unique challenges such as choice of test species and integration of SP parameters into repeat-dose toxicity studies. This chapter reviews the evolution of SP assessment of BPs using the approval packages of marketed BPs and discusses the past, current, and new and upcoming approach and methods that can be used to generate high-quality data for the assessment of SP of BPs.

Keywords

Biopharmaceuticals • Cardiovascular system • Central nervous system • International Conference on Harmonization • Respiratory system • Safety pharmacology

1 Introduction

Biotechnology-derived pharmaceuticals or biopharmaceuticals (BPs) are molecules such as monoclonal antibodies (mAb), soluble/decoy receptors, hormones, enzymes, cytokines, and growth factors that are produced in various biological expression systems and are used to diagnose, treat, or prevent various diseases (International Conference on Harmonization (ICH) S6(R1) 2011). Other types of novel therapeutics, such as vaccines and oligonucleotides synthesized by bacterial or mammalian cells, are also considered BPs according to the ICH S6 (R1) guideline.

The features of BPs that distinguish them from traditional small molecule (SM) therapeutics are their relative larger physical size and molecular weight (typically $<$500 Da for SMs and $>$1,000 Da for BPs), molecular complexity, and unique selectivity for the intended therapeutic target. Because of this latter characteristic, BPs are expected to have less off-target activities relative to SM therapeutics and consequently have a reduced risk of off-target adverse effects in humans (Giezen et al. 2008; Amouzadeh and Vargas 2013). Important properties of BPs and SM therapeutics are listed in Table 1.

Innovative variations in the engineering of BPs have been developed in an effort to create new therapeutics to treat human disease. For example, peptibodies, which are peptides fused to an IgG Fc molecule, have emerged. The only marketed peptibody, romiplostim (NPLATE®), was approved under a Biologic License Application (BLA) by the United States Food and Drug Administration (FDA) in 2008 and by European Medicines Agency (EMA) in 2009 for the treatment of immune thrombocytopenic purpura (Shimamoto et al. 2012).

Table 1 Comparison of general characteristics of small molecules and biopharmaceuticals

Attribute	Small molecules	Biopharmaceuticals
Modality	Synthetic chemicals	mAb, peptides, peptibodies, fusion proteins, ADC, BiTEs®, and vaccines
Synthesis	Chemical	Biotechnological
Physiochemical properties	Well-defined, single molecule	Complex, heterogeneous
Molecular mass	<500–1,000 Da	>1,000 Da
Stability	Stable	Sensitive to heat and shear
Target selectivity	Low to high	High
Typical route	Oral	Parenteral
Distribution	Widespread	Plasma and extracellular space
Metabolism	• Inactive and active metabolites • CYP inhibition/induction • Covalent binding	Amino acids
Half-life	Short (<24 h)	Long (days to weeks)
Disposition	Linear or nonlinear PK	PK: altered by ADA and TMDD
Bioanalytical methods	LC/MS	Bioassay
Drug–drug interaction	High—both PK and PD	Low—mostly PD
Immunogenicity	Rare	Possible
Regulatory guidelines	ICH M3, S7A, and S7B	ICH S6R1, S7A and B
Typical species of choice	Rodent and non-rodent	NHP
Safety pharmacology Core battery	Dedicated	Integrated into toxicity studies
QT liability	• hERG assay • QT_c assay (non-rodent)	QT_c assay (non-rodent)
Toxicity	"On-/off-target"	"On-target" (exaggerated pharmacology)

mAb monoclonal antibody, *ADC* antibody-drug conjugates, *BiTEs* bispecific T cell-engaging antibodies, *Da* dalton, *CYP* cytochrome P-450, *PK* pharmacokinetic, *ADA* anti-drug antibody, *TMDD* target-mediated drug disposition, *LC/MS* liquid chromatography/mass spectroscopy, *PD* pharmacodynamic, *ICH* international conference on harmonization, *NHPs* nonhuman primates

Other examples of modified BPs are the antibody-drug conjugates (ADCs), which are used primarily as oncology therapeutics. These particular molecules are mAb-SM drug hybrids that are designed to take advantage of the selectivity of mAbs to deliver small cytotoxic molecules to specific tumor cells (Perez et al. 2014). Three ADCs have been approved by FDA thus far. These include gemtuzumab ozogamicin (MYLOTARG®) which targets CD33 for acute myelogenous leukemia, brentuximab vedotin (ADCETRIS®) which targets CD30 for Hodgkin lymphoma and anaplastic large cell lymphoma, and ado-trastuzumab emtansine (KADCYLA®) which targets HER2 positive metastatic breast cancer (Drugs@FDA). Although all of these molecules are classified as ADCs, it is

noteworthy that the first was reviewed by the FDA under a New Drug Application (NDA) as an SM, and the latter two were reviewed as BLAs (Drugs@FDA).

A more recent class of novel BPs in clinical development is bispecific T cell-engaging antibodies (BiTE®). Bispecific antibodies, in general, are engineered to recognize two distinct epitopes. BiTE® antibodies are comprised of two flexibly linked single-chain variable fragments of different antibodies, one directed against a tumor antigen and one targeting CD3 on T cells. As a result, these bispecific antibodies can transiently link tumor cells with resting polyclonal T cells to induce a surface target antigen-dependent redirected lysis of tumor cells. This pharmacological action closely mimics the natural cytotoxic T cell response and leads to the selective destruction of cancer cells. Blinatumomab (targets CD19 antigen on B cells) and solitomab (targets the epithelial cell adhesion molecule (EpCAM) antigen) are examples of BiTE®s for treatment of blood, lung, and gastrointestinal (GI) cancers, respectively (Frankel and Baeuerle 2013). The first BiTE® antibody, blinatumumab (BLINCYTO) BLINCYTO®, was approved in 2014 under accelerated approval program for the treatment of Philadelphia chromosome-negative relapsed or refractory B cell precursor acute lymphoblastic leukemia (Drugs@FDA).

The first BP, recombinant insulin, was approved in 1982 (Marafino and Pugsley 2003). Since then, there has been an increasing number of BPs approved for various indications. By the end of 2014, a total of 111 novel BPs have been approved by the FDA (Drugs@FDA). A survey of new drug approval trends (1994–2004) indicated that BPs have a better chance of attaining regulatory approval than conventional SM therapeutics (32 % vs. 12 %, respectively; DiMasi et al. 2010). This is due to low success rate in developing SMs for discrete targets such as the central nervous system (CNS), which has an 8 % success rate and a lower rate of attrition among BPs (DiMasi et al. 2010; Giezen et al. 2008). The latter is supported by recent data showing a withdrawal rate of 5 % for BPs and 9 % for SMs during 1998–2008 (DiMasi et al. 2010). Three BPs, efalizumab (RAPTIVA®; FDA 2009), gemtuzumab ozogamicin (MYLOTARG®; FDA 2010a), and peginesatide (OMONTYS®; FDA 2013a) were withdrawn from human use because of increased risk of progressive multifocal leukoencephalopathy (a rare and usually fatal disease caused by activation of the JC virus by a combination of pharmacological agents and immune compromise), lack of efficacy and safety concerns, and serious hypersensitivity reactions, respectively.

2 Safety Pharmacology Evaluation of Biopharmaceuticals: A Changing Landscape

Safety pharmacology (SP) assessment of BPs has evolved since the approval of recombinant human insulin in 1982. A recent review of the FDA approval packages for BLAs demonstrated diverse approaches towards SP assessment of BPs. Among the 111 BPs that have been approved (1980–2014: BLA: 110; NDA: 2), a variety of SP assessment strategies have been used. Among these BLA packages, 27 had no

specific pharmacology/toxicology reviews, 32 indicated that no SP studies were performed, 21 had SP evaluation integrated into toxicity studies, and 31 had dedicated SP studies. Overall, 62 % of the BLAs with a reported nonclinical safety summary cited SP information collected from dedicated or integrated studies (Table 2). However, many of the integrated and dedicated SP studies did not include all the required cardiovascular, CNS, respiratory assessments, i.e., core battery (Table 2). An important conclusion from this retrospective review is that various SP approaches have been applied in the evaluation of approved BPs. This is perhaps due to the fact that regulatory guidelines are intentionally not prescriptive, to allows sponsors discretion in the nonclinical safety assessment strategy used for their unique drug candidate and to accommodate innovative methods and approaches such as telemetry and integrated study design, e.g., the capture of SP functional endpoints in toxicity studies.

The evolution of SP, and toxicology practices for BPs, is ongoing and will be influenced by many factors, including (1) the novel scientific attributes and liabilities of new BPs associated with their mechanism of action and molecular targets, (2) the emergence of new methods or technologies to improve SP assessment, and (3) the ability to integrate "fit for purpose" functional evaluations in repeat-dose toxicity studies. The opportunities to introduce quality SP assessments into toxicity studies, as well as the pitfalls to consider, are highlighted in an excellent review article by Redfern et al. (2013). A key limitation of typical SP studies is that they are designed primarily as acute (single-dose) experiments, so functional effects that intensify or diminish (due to tolerance) with longer exposure are not evaluated systematically. This limitation represents a gap in the ability to perform clinical risk assessments based on functional hazards that occur with chronic dosing. This safety assessment gap can be mitigated by introducing sensitive SP evaluations into repeat-dose toxicity studies or performing dedicated repeat-dose SP studies. The changing landscape of SP evaluation for BPs and SMs is underscored by a recent pharmaceutical industry survey, which reported that many

Table 2 A survey of safety pharmacology information for novel BPs approved by US-FDA from 1980 to 2014

Modality	BLA[a]	Dedicated		Integrated		None	No data
		Core	Partial	Core	Partial		
Antibodies	41	2	6	4	11	9	10
Proteins/peptides	30	7	7	1	3	9	4
Enzymes	20	4	4	2	0	8	6
Cytokines	16	2	1	0	0	6	7
ADC	3	1	2	0	0	0	0
BiTE®	1	1	1	0	0	0	0
Total	111	17	14	7	14	32	27

Dedicated: Specific cardiovascular, neurobehavioral, and respiratory studies conducted
Integrated: SP endpoints were collected in toxicology studies
Core: all three assays; partial: only one or two assays; ADC: antibody-drug conjugate
[a]2 BP were approved as NDA: pasireotide (peptide) and gemtuzumab ozogamicin (ADC)

drug sponsors are actively using improved functional methods, like jacket-based telemetry systems for noninvasive cardiovascular (CV) monitoring, to detect functional effects after acute and chronic treatment in exploratory or Investigational New Drug (IND)-enabling toxicity studies (Authier et al. 2013).

3 Regulatory Guidelines

The overall goal of guidelines addressing the SP profiling of new therapeutics is to assure human safety upon first administration of novel drug candidates, and beyond. These guidelines include the general approaches on the nonclinical safety evaluation of drug candidates. Because of the unique nature of each drug candidate, either SM or BP, its safety assessment should be based on sound scientific rationale. This will allow for adequate characterization of the safety profile of a drug candidate prior to first administration to humans.

Safety pharmacology assessment of BPs is based on ICH M3(R2) (2009), S6 (R1) (2011), S7A (2000), and S7B (2005) guidelines. With regard to BPs, the M3 (R2) guideline addresses only the "timing of nonclinical studies relative to clinical development" and defers to S6 guideline for nonclinical safety assessment of biotechnology-derived drugs. The S6(R1) guideline indicates that "It is important to investigate the potential for undesirable pharmacological activity in appropriate animal models and, where necessary, to incorporate particular monitoring for these activities in the toxicity studies and/or clinical studies" (Section 4.1, ICH S6 (R1) 2011). Safety pharmacology assessment of BPs should be designed to reveal the potential adverse effects of the drug candidate on the function of CV, central nervous, and respiratory systems. Guidelines S7A and S7B specifically indicate that SP evaluation of BP can be performed using dedicated studies or an integrated approach where SP parameters are measured in toxicity studies, including QTc prolongation evaluation (a surrogate for assessment of torsades de pointes or pro-arrhythmic liability).

The potential undesirable effect of every drug candidate on the CV, central nervous, and respiratory systems, referred to as the "core battery" (ICH S7A), should be assessed prior to first-in-human administration. In addition, follow-up studies may be needed to allow for better understanding of the effects of drug candidates on these systems. Supplemental SP studies may also be needed to assess the effects of drug candidates on other systems such as renal and gastrointestinal (GI), if effects on these systems are suspected based on the class of the drug candidates being tested and/or target liability assessment, e.g., target expression in the particular organ. The core battery studies should be performed in compliance with the good laboratory practice (GLP); however, "follow-up and supplemental studies should be conducted in compliance with GLP to the greatest extent feasible" (Section 2.11, ICH S7A 2000). These SP studies can be either stand-alone or SP parameters assessment could be integrated into toxicity studies. However, the interrogation of SP parameters in a toxicity study should follow the same standards applied to dedicated studies, according to a recent FDA Guidance for Industry (Questions and Answers on ICH M3(R2) Guideline) (Section E, FDA 2013b).

There are circumstances where SP assessment may not be required. These include therapeutics being developed for topical application (dermal or ocular) where the systemic exposure is expected to be low (Section 2.9, ICH S7A 2000). In addition, novel cytotoxic agents being developed for advanced cancer do not require dedicated SP studies, unless there is a cause for concern (Section II B, ICH S9 2010).

Although ICH S6(R1) guideline principals may be applicable to recombinant DNA protein vaccines, SP evaluation of vaccines is not required based on the recommendation of the World Health Organization (WHO) unless "nonclinical and/or human clinical studies suggest that the vaccine my affect physiological functions" (WHO 2003). In that case, SP parameters should be incorporated into toxicity studies (WHO 2003; 2013). This recommendation has been adopted by the FDA (Sun et al. 2012); however, European Union and Japanese regulatory agencies recommend that the effects of vaccines on CV, central nervous, and respiratory systems functions be assessed during repeat-dose toxicity studies before first-in-human exposure (Sun et al. 2012). There are not many reports that describe the SP evaluation of a vaccine, but a report on the activity of an experimental protein-based cancer vaccine [CHP-NY-ESO-1 peptide vaccine—consisting of a recombinant protein of the cancer antigen NY-ESO-1 and a polysaccharide-based delivery system (cholesteryl pullulan)] does provide some insight. In a traditional battery of in vitro and in vivo SP studies, the results indicated that the vaccine did not inhibit hERG channel function and had no effect on vital functions after acute administration, which indicated that this vaccine product had very low potential for altering the CV, central nervous, and respiratory systems (Harada et al. 2008).

For detailed information on safety assessment of vaccines (prophylactic and therapeutic), readers are referred to reviews on this topic (Lebron et al. 2005; Sun et al. 2012; Matsumoto et al. 2014). SP for bacterial- or mammalian cell-derived oligonucleotides is typically evaluated during repeat-dose toxicity studies (Dixit et al. 2010; Kim et al. 2014); however, recommendations and strategies that can be used to assess these unique agents have been developed by the SP subcommittee of the Oligonucleotide Safety Working Group (Schubert et al. 2012; Berman et al. 2014).

4 Choice of Test Species

Choice of the test species is a critical step in the evaluation of the nonclinical safety of BPs. The most important factor in choosing a test species is whether the intended therapeutic target (receptor, channel, etc.) is present in a given species and whether the molecular identity and transduction mechanisms of the target is similar to that in humans. SP assessment of SMs is performed in rodents and non-rodents (Table 1). In contrast, SP assessment of BPs in rodent may not be possible because rodents do not always express orthologue of the human target, or BPs have little or no pharmacological activity against the rodent orthologue. For this reason, nonhuman

primates (NHPs) are the primary species for assessing the safety of BPs (Bussiere 2008). This is supported by a recent pharma-wide survey of SP practices (Authier et al. 2013).

5 In Vitro Safety Pharmacology Profiling

In vitro SP profiling of SM drug candidates, using either binding or cellular functional assays, is typically performed early in the drug development process to allow for selection of a drug candidate with lower potential for off-target activity or side-effect liability (Bowes et al. 2012). Because of the high target selectivity, BPs are not expected to have significant off-target activities; thus, these agents are not typically profiled for secondary pharmacological activity. Two current examples that support the hypothesis that BPs have low off-target potential were identified in an examination of drug approvals. In our survey of approved BPs (Table 2), we found that one peptibody and one monoclonal antibody were profiled pharmacologically for off-target activity. Using conventional methods, romiplostim (NPLATE®, 59,000 Da) and adalimumab (HUMIRA®, ~148,000 Da) were tested in panels containing 63–68 receptors, enzymes, and ion channels and found to be devoid of any significant "off-target" activity, which indicates that these agents were highly selective for their molecular targets.

We reported the receptor profile of an investigational pegylated (polyethylene glycol-20) peptide (~24,000 Da) that was evaluated for potential off-target interactions with 151 receptors, enzymes, ion channels, and transporters. The results indicated eight hits (a "hit" being defined as >50 % inhibition of control-specific binding or enzyme activity at 10 μM), but these were not considered to have relevant safety implications because of their high-exposure multiples relative to the human target potency estimates (Vargas et al. 2013a). In addition, when the PEG moiety alone was tested in the same panel, no significant off-target hits were observed. Based on the current state of knowledge and regulatory practices, in vitro pharmacology profiling of the BPs is not recommended.

6 Cardiovascular System Safety Pharmacology

The nonclinical approach for CV risk assessment of new drug candidates should address potential effects on blood pressure, heart rate, electrocardiography (ECG), and the functional status of critical ion channels (ICH 7A 2000; ICH 7B 2005). To achieve this, various in vitro and in vivo methods can be used. In general, the approach for BPs and SMs is similar; however, there are differences. The first parameter evaluated in the CV risk assessment of SMs is their potential inhibitory effect on hERG channel function using a voltage-clamp assay. Performing this assay for BPs is not considered appropriate because large proteinaceous molecules are not expected to pass though the plasma membrane to gain access to the channel pore, nor are they likely to interact with the "toxin-binding site" on the

extracellular surface of the hERG channel. Recent in vitro studies using anti-hERG-specific antibodies show that these antibodies do not inhibit the function of the channel because they do not bind to key epitopes near the external pore region like BeKm-1 (Qu et al. 2011). Therefore, performing a hERG assay with BPs is considered irrelevant and is not recommended (Vargas et al. 2008).

Blood pressure, heart rate, and the ECG can be assessed in conscious freely moving rodents, dogs, and NHPs using telemetry. For SMs, which typically have a short half-life, this is done using a crossover design where each animal receives every treatment and serves as its own control in a single-dose study. This study design is not appropriate for BPs because of their long half-life. For this reason, integration of CV parameters into repeat-dose toxicity studies with BPs is an appropriate approach which also has the advantage of reducing the overall number of animals. Jacket-based external telemetry (JET™) is the method of choice for collection of high-quality heart rate and ECG parameters from unrestrained animals, particularly NHPs, which are often the species of choice for safety assessment of BPs (Chui et al. 2009, 2011; Guth et al. 2009; Derakhchan et al. 2011, 2014). Performed appropriately, this method allows for collection of parameters during non-rodent toxicity studies that are of comparable quality to those collected in unrestrained large animals using implant telemetry (Chui et al 2009; Derakhchan et al. 2014). The critical aspect of JET™ is the acclimation of the animals to the jacket, which is especially important for NHPs. The acclimation process generally involves multiple sessions of increasing time to allow the animals to become accustomed to the jacketing process and to wearing the jacket. Because acclimation is not long-lasting, it should be conducted as close to the collection period as possible (Derakhchan et al. 2014).

For BPs, hemodynamic parameters such as arterial blood pressure can be assessed using implant telemetry in NHPs (Santostefano et al. 2012). Alternatively, arterial pressure can be measured directly using JET™ with the blood pressure option (JET-blood pressure; McMahon et al. 2010). Implanted telemetry has the advantage of not requiring acclimation to handling and wearing of a jacket, but time is needed to allow the animals to recover from the implantation surgical procedure. Either method (implant telemetry or JET-blood pressure) allows for the collection of arterial pressure data from unrestrained animals for short or long durations which are well suited to characterize the hemodynamic profile of BPs (McMahon et al. 2010; Kaiser et al. 2010). Another method under development for measurement of blood pressure is high-definition oscillometry (Schmelting et al. 2009). At this time, there are many issues with this method, which may limit its usefulness as a sensitive SP tool. These include the inability to detect small changes in blood pressure, inaccurate blood pressure and heart rate due to stress from restraint, and limitation to a single-point measurement as compared to continuous measurements provided by other methods (Kurtz et al. 2005; Wernick et al. 2012).

Cardiac function can also be evaluated directly using echocardiographic imaging (Tsusaki et al. 2005; Hanton et al. 2008) or by measuring left ventricular pressure as an index of cardiac inotropy (Sarazan et al. 2011). The main advantage of the echocardiography method is that the same functional and structure parameters can

be evaluated in both animals and humans noninvasively, which allows for direct comparison of the same endpoints and their translation across species. However, unlike continuous monitoring of left ventricular function by telemetry in freely moving conscious animals, echocardiography is a "snapshot" measurement, i.e., at a single time point, which may require chemical restraint (or extensive acclimation of animals if chemical restraint is not used) to ensure the animals remain properly positioned for the time required to obtain quality cardiac images.

There are cases when a dedicated CV telemetry study may be the best option to assess target liability concerns for BPs. These include the presence of the therapeutic target in the CV system (e.g., cardiac myocytes, vascular endothelium, or vascular smooth muscle) or the emergence of CV findings in nonclinical toxicity studies or clinical trials. For example, observation of cardiac dysfunction in patients treated with trastuzumab, a mAb for treatment of breast cancer, prompted the sponsor to perform a long-term telemetry study in the cynomolgus monkey in attempts to model the human cardiac dysfunction (Klein and Dybdal 2003). Likewise, a novel ADC based on trastuzumab was evaluated in a dedicated NHP telemetry study to evaluate potential target-mediated CV effects (Poon et al. 2013).

In general, the risk for QTc prolongation is considered low for BPs. This is supported by the findings from a review of FDA drug approvals from 2008 to 2015 (Hughes 2009, 2010; Mullard 2011, 2012, 2013, 2014, 2015). Among the 57 BPs approved during this period, only 2 BPs had a QT warning on their label (3.5 %; Table 3). In contrast, 17 of 159 SMs approved during the same period had a QT warning (10.6 % Table 4). Likewise, 93 thorough QT (TQT, a dedicated clinical study designed to assess drug-induced changes in the QTc interval) studies were performed as part of the registration requirements for SMs, compared to only 4 TQT studies on a few BPs (e.g., peginesatide acetate, pasireotide, albiglutide, and ramucirumab).

As part of their clinical development, peginesatide acetate, albiglutide, and ramucirumab were assessed for QTc prolongation risk in a valid TQT study, i.e., with a positive control, and each drug had no effect on cardiac repolarization in humans (peak effect: <10 ms). However, pasireotide diaspartate did have a positive QTc signal, which resulted in a QT warning on the label (Drugs@FDA). Given that BPs are generally considered to have a low risk for cardiac ion channel blockade and QTc prolongation, the effect following pasireotide diaspartate administration deserves further review.

The QTc prolongation caused by pasireotide diaspartate may be an example of a target-driven cardiac safety concern. This drug is a synthetic peptide analog of somatostatin and used for treatment of Cushing's disease (Mullard 2013; Drugs@FDA). Nonclinical studies, including hERG function ($IC_{50} > 30$ μM), Purkinje fiber assays, and a NHP telemetry study demonstrated low potential for delayed cardiac repolarization. Despite the nonclinical profile, QTc prolongation was observed in two TQT clinical trials with this agent. The findings from the initial trial were not reported; however, in the pivotal trial, QT prolongation was reported in approximately 6 % of subjects (Clinical Summary, Drugs@FDA). In healthy volunteers given pasireotide subcutaneously, the mean ΔΔQTcI was 13.2 and

Table 3 Biopharmaceuticals (BPs) with a TQT study or a QT warning in the label

Year	Approved drugs	Approved BPs	TQT study	TQT signal	QT warning
2008	24	4	0	0	1
2009	27	7	0	0	0
2010	21	9	0	0	0
2011	35	11	0	0	0
2012	39	11	2[a]	1	1
2013	27	4	0	0	0
2014	41	11	2	0	0
Total	214	57	4	1	2

TQT: thorough QT
[a]These studies were performed on one BP (pasireotide)

Table 4 Small molecule (SM) therapeutics with TQT studies and QT warning in the label

Year	Approved drugs	Approved SMs	TQT study	TQT signal	QT warning
2008	24	20	11	2	2
2009	27	20	7	2	7
2010	21	12	4	1	1
2011	35	24	16	2	3
2012	39	28	15	1	2
2013	27	25	16	0	0
2014	41	30	24	2	2
Total	214	159	93	10	17

TQT: thorough QT

16.1 ms for the 600 and 1,950 μg twice-daily doses, respectively (Breitschaft et al. 2014). As there was no nonclinical evidence for a direct inhibitory effect of pasireotide on the hERG channel or evidence of any change in Purkinje fiber APD, the specific mechanism of the QTc effect is unknown; however, it could be an indirect or secondary effect on cardiac repolarization. For example, pasireotide administration was associated with hyperglycemia and bradycardia, so the delayed cardiac repolarization could be due to treatment-related alterations in autonomic tone and glucose modulation. Another somatostatin analog, octreotide, has also been associated with QTc prolongation (Drugs@FDA), potentially through a similar mechanism as pasireotide.

Other BPs, such as oxytocin, have been associated with cardiac repolarization risk (see crediblemeds.org). Adverse CV effects, including hypotension, elevated heart rate, cardiac arrhythmia, premature ventricular contractions, and QTc prolongation, have been observed in women treated with oxytocin during abortion and cesarean delivery (Charbit et al. 2004; Guillon et al. 2010). In a nonclinical investigation, similar QTc findings were reported in rabbits administered intravenous bolus injection of oxytocin (Uzun et al. 2007). A mechanistic study of recombinant oxytocin on repolarization in rabbit and human ventricular myocytes demonstrated that this peptide did not inhibit hERG channel function or prolong

action potential duration (APD) and QTc intervals in cardiac models, which strongly suggests that the QTc prolongation observed in humans and animals is not mediated through a direct cardiac site of action (Vargas et al. 2013b; Qu et al. 2015). The findings with pasireotide and oxytocin indicate that some BPs can alter cardiac repolarization indirectly; thus, there is a potential for drug–drug interactions with other therapeutics that have QTc prolongation risk (e.g., antiarrhythmics, some antibiotics, etc.).

7 Central Nervous System Safety Pharmacology

Assessment of the effects of drug candidates on CNS function is required prior to first-in-human exposure for both BPs and SMs (ICH S7A 2000). However, the approach for each type of drug modality is different. For SMs, neurobehavioral effects are typically assessed in rodents using neurofunctional methods such as Irwin, modified Irwin, or a functional observational battery (FOB) which include a battery of observational parameters such as home cage and open arena observation and elicited responses such as reflexes to stimuli. BPs are not expected to enter the CNS under normal circumstances because of their physiochemical properties such as large molecular size and the unique features of the bloods brain barrier (BBB) which is designed to prevent chemicals from gaining access to the brain (Misra et al. 2003; Gabathuler 2010; Freeman et al. 2012; Pardridge 2012). Therefore, it is generally accepted that, under normal physiological conditions, BPs are not expected to enter the CNS and affect neurobehavioral function. This notion is supported by the results of an internal survey of incidence of convulsion which indicates that none of the BPs ($N = 11$) caused convulsion whereas 14 % of small molecules caused convulsion. The incidence of convulsions was further confirmed by electroencephalographic studies in either rats or in NHPs which showed evidence of seizure activity for SMs that cause convulsion (Amouzadeh and Vargas 2013; Vargas et al. 2013a). In addition, the label for raxibacumab, a mAb against protective antigen of *Bacillus anthracis* for prophylaxis or treatment of inhalational anthrax in combination with antibacterial drugs, explicitly indicates that it "appears unable to penetrate the CNS until compromise of the BBB during latter stages of anthrax infection" (Drugs@FDA).

To overcome the lack of access of the BPs to the CNS in cases such as brain tumors where a BP could be a beneficial therapy, drug-delivery approaches such as receptor-mediated transcytosis through BBB can be exploited to deliver BPs to the CNS (Yu et al. 2011; Pardridge and Boado 2012; Yi et al. 2014).

Regardless of theoretical and practical aspects, neurobehavioral effects of BPs should be evaluated prior to first-in-human exposure. In cases where the therapeutic target is present in rodents, CNS studies can be performed either in dedicated Irwin/FOB studies or parameters can be integrated into toxicity studies. However, when the choice of species is limited to NHPs, neurobehavioral effects of BPs are typically assessed during repeat-dose toxicity studies as recommended by ICH 7A (2000). The use of an integrated approach has the advantage of being in line

with 3Rs principles, especially the optimization of NHPs use in toxicity studies. Methods have been developed to obtain detailed neurobehavioral data from NHPs beyond basic clinical observations as part of standard toxicity or cardiovascular telemetry studies (Korte et al. 2007; Gauvin and Baird 2008; Moscardo et al. 2010).

A recent report showed that assessing spontaneous locomotor activity in rodents using noninvasive methods based on infrared beam (Actimeter®) or vibration (LABORAS®) is a useful predictor of neurobehavioral effects of drug candidates (Lynch et al. 2011; Golozoubova et al. 2014) that can be used early in the drug development process. Although BPs are not expected to cause significant neurobehavioral effect because of their poor penetration into the CNS under normal circumstances, the possibility of neurobehavioral effects cannot be ruled out. A recent survey of 50 drug candidates for non-CNS indications (and with limited access to CNS) showed that the majority of them affected at least one parameter of the FOB in rats. However, this may not necessarily mean a specific effect on the CNS, but rather secondary behavioral changes due to general drug-induced toxicity or CV effects (Redfern et al. 2005). Therefore, observation of an effect during the neurofunctional evaluation of a BP should be followed by more rigorous testing to determine whether the effect was actually caused by action of the BP on the CNS or an indirect behavioral effect.

8 Respiratory System Safety Pharmacology

Because of their selectivity, BPs are not expected to adversely affect the respiratory system through their potential off-target activity, unless there is a concern about target-based toxicity. This is reflected in a recent publication documenting a lack of adverse respiratory effects in the rat with BPs (Vargas et al. 2013a). However, assessment of respiratory function in nonclinical species is required prior to first-in-human exposure. ICH 7A (2000) guideline states that the "Effects of the test substance on the respiratory system should be assessed appropriately. Respiratory rate and other measures of respiratory function (e.g., tidal volume or hemoglobin oxygen saturation) should be evaluated. Clinical observation of animals is generally not adequate to assess respiratory function, and thus these parameters should be quantified by using appropriate methodologies" (Section 2.7.3, ICH 7A 2000). Based on the findings from the initial assessment, there may be a need to perform follow-up studies to evaluate "airway resistance, compliance, pulmonary arterial pressure, blood gases, blood pH, etc." (Section 2.8.1.3, ICH 7A 2000).

Typically, respiratory system SP assessments of SMs are performed using either head-out or whole-body plethysmography. Parameters such as respiratory rate (RR) and tidal volume (TV) are measured, and many other parameters such as minute volume, peak inspiratory flow (PIF), peak expiratory flow (PEF), enhanced pause (Penh), inspiration time (IT), and expiration time (ET) are derived. Although there are many methods for respiratory function assessment in rodents (Murphy 2002, 2014; Hoffman et al. 2007; Hoymann 2012), such methods have not been validated extensively for large animals such as NHPs. Therefore, respiratory

function assessment of large animals is integrated in toxicity studies and oftentimes is limited to clinical observation during toxicity studies which is not a sensitive method to assess subtle changes in respiratory function. Although respiratory function assessment could be performed during a toxicity study, it requires skilled staff and careful planning to assure that accurate data are collected during maximal drug effect. There may be cases where a more rigorous assessment of the respiratory function such as pulmonary resistance is needed based on the presence of therapeutic target in the lung and/or empirical observation of pulmonary (lung) pathology in toxicity studies of a BP drug candidate. For this, a dedicated respiratory SP study may be needed to assess functional consequences and inform the need for a clinical monitoring strategy. Initial assessment can be performed in NHPs using head-out or whole-body plethysmography (Iizuka et al. 2010; Lawler et al. 2006; Nalca et al. 2010), and follow-up evaluation, such as measurement of pulmonary resistance, can be done in anesthetized animals (Chapman et al. 2005; Skeans et al. 2005; Curran et al. 2008). Recently, airway oscillometry has been reported to be a useful method for noninvasive evaluation of respiratory function in dogs and cynomolgus monkeys (Bassett et al. 2014b). This latter method holds promise to enable a more robust and quantitative evaluation of respiratory function in non-rodent SP and toxicology studies.

9 Follow-Up and Supplemental Safety Pharmacology

Follow-up and supplemental SP studies may be needed based on the findings from "safety pharmacology core battery, clinical trials, pharmacovigilance, experimental in vitro or in vivo studies, or from literature reports" (ICH 7A 2000).

The purpose of follow-up studies is to allow for a further understanding of the findings. These follow-up studies could include "behavioral pharmacology, learning and memory, ligand-specific binding, neurochemistry, visual, auditory and/or electrophysiology examinations, cardiac output, ventricular contractility, vascular resistance, the effects of endogenous and/or exogenous substances on the cardiovascular responses, airway resistance, compliance, pulmonary arterial pressure, blood gases, blood pH, etc." (ICH 7A 2000).

The need for the conduct of supplemental SP studies is informed by findings during nonclinical development when adverse effects on the function of systems other than those in the core battery are observed. These systems include renal/urinary, autonomic, GI, and other organ systems. ICH S7A guideline cites a number of parameters that can be used to assess the effects of drug candidates on the function of these systems. For example, urinary parameters such as volume and electrolyte excretion, GI parameters such as transit time and diarrhea, immunological parameters such immune cell phenotyping, and endocrine parameters such as hormone levels may be used to assess functional changes in these particular organ systems (Section 2.8.2, ICH 7A 2000). These parameters could be assessed either in stand-alone SP or repeat-dose toxicity studies. For example, stand-alone video-EEG can be used to assess seizure liability in rats, dogs, or monkeys (Bassett

et al. 2014a). The approaches for SP assessment of SMs and BPs described above apply to the follow-up and supplemental pharmacology studies as well; thus, supplemental SP endpoints can be evaluated as add-on measurements as part of a repeat-dose toxicity study.

Assessment of abuse and dependency potential of a drug candidate is also needed if it shows CNS activity and is chemically or pharmacologically similar to known drugs of abuse and produces psychoactive effects (Section 15, ICH M3 (R2) 2009). The general nonclinical approach and details for this assessment are described in the guidance issued by FDA (FDA 2010b), the decision tree presented by FDA (Bonson and Sun 2011), and the guideline issued by EMA in 2006. Based on the rationale and empirical findings cited above on the lack of access to CNS by BPs, assessment of abuse and dependency potential of BPs are not warranted. However, in cases where a BP is designed to penetrate the CNS, such assessment may be needed (Yu et al. 2011; Pardridge and Boado 2012).

10 Summary

Safety evaluation of novel BPs begins during target liability assessment when potential adverse effects of modulating a target are identified. The most efficient path to SP evaluation of novel BPs and fulfillment of the regulatory requirements is by integrating the collection of relevant SP parameters into toxicity studies when possible and practical (Redfern et al. 2013; Authier et al. 2013). In specific cases, based on target liability concerns, or the emergence of unanticipated pharmacological or toxicological findings, a dedicated SP study may be needed to address the liability (Santostefano et al. 2012; Klein and Dybdal 2003; Poon et al. 2013). There may also be a need to perform supplemental SP when there is a cause for clinical concern. Thus, the core message is that the SP strategy for BPs will be influenced by many factors and is guided by the need to know whether functional changes in organ systems responsible for vital functions (CV, central nervous, and respiratory systems) are impacted by a BP. In vitro SP profiling of the BPs is not recommended as part of routine screening based on the current state of knowledge and regulatory practices.

11 Future Prospects

The safety assessment of drug candidates is an evolving science. This has been envisaged in the nonclinical guidelines in that they recommend general, rather than specific, approaches to safety assessment. This is because the development of each BP drug candidate is unique (or "fit for purpose") and there is continued improvement in the understanding of the science, technologies, and methods during preclinical drug development.

One of the major improvements in nonclinical SP assessment of the drug candidate is the use of telemetry systems which allow for extended evaluation of

CV parameters in unrestrained rodents and non-rodents. An example is the evaluation of the effects of drug candidates on the CV system using either a jacket or an implanted device to collect high-quality ECG or blood pressure data for extended periods.

There are also emerging methodologies for improved assessment of respiratory, central nervous, GI, and renal systems. However, many of these methods are not part of standard practice, because they have not been widely studied or validated. Jacket-based inductive plethysmography (Ingram-Ross et al. 2012) and airwave oscillometry (Bassett et al. 2014b) are noninvasive methods that can be used to monitor respiratory function quantitatively in large animals in either stand-alone or integrated SP studies. Assessing spontaneous locomotor activity in rodents using noninvasive methods based on infrared beam (Actimeter®) or vibration (LABORAS®) has been shown to be a useful predictor of neurobehavioral effects of drug candidates (Lynch et al. 2011). This technique can be used to evaluate the potential of BPs to cause neurobehavioral effects early in the drug development process if the target is expressed in the test species. New technologies such as SmartPill® may be useful to assess effects of BPs on GI function (transit time), and biomarkers of dysfunction (e.g., blood urea nitrogen or creatinine) or injury (e.g., KIM-1) could be used as an indicator of renal toxicity of BPs, but will require further testing and validation in large animal species, especially the NHPs.

An alternative approach to CNS and respiratory SP assessment in rats is performing these studies using the same animals, but tested in a sequential manner. During the first phase of the study, neurobehavioral effects of the test compound are assessed. Then, an interim period of at least 1 week is allowed for clearance of the test compound during which animals are gradually acclimated to the plethysmography apparatus. During the second phase, the effect of test compound on the respiratory system is evaluated. This alternative approach is most appropriate for SMs, which typically have a short half-life or BPs with short half-life such as peptides that show activity in rodents. Major advantages of this approach are substantial reduction in the number of animals and the potential for lower overall study costs.

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Safety Pharmacology of Anticancer Agents

Pauline L. Martin

Contents

Abstract

The safety pharmacology testing for anticancer agents has historically differed for small molecule pharmaceutical drugs versus large-molecule biopharmaceuticals. For pharmaceutical drugs, dedicated safety pharmacology studies have been conducted according to the ICH M3 (R2), ICH 7A, and ICH S7B guidance documents. For biopharmaceuticals, safety pharmacology endpoints have been incorporated into the repeated-dose toxicology studies according to ICHS6 (R1). However, the introduction of the ICH S9 guidance document for the nonclinical evaluation for anticancer pharmaceuticals has allowed for a streamlined approach for both types of molecules to facilitate access of new potential therapeutics to cancer patients and to reduce the number of animal studies. Examples of the testing strategies that have previously been employed

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for some representative anticancer agents are provided, and their predictivity to adverse events noted in the clinic is discussed.

Keywords
Anthracycline • Anticancer • Biopharmaceutical • Monoclonal antibody • Pharmaceutical • Regulatory guidance • Tyrosine kinase

1 Introduction

Nonclinical safety assessments of anticancer pharmaceuticals generally include repeated-dose toxicity testing in two species (a rodent and a non-rodent, most frequently rat and dog) with in-life evaluations of safety as well as postmortem examination for any macroscopic or microscopic pathological changes (International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) harmonized tripartite guideline ICH M3 (R2) 2009). In addition, a series of dedicated safety pharmacology studies may be conducted to evaluate potential adverse pharmacodynamic effects on major organ systems that may not be detected in the general toxicity studies (Pugsley et al. 2008). The "core battery" of tests includes an evaluation of potential adverse effects on the central nervous system (CNS), cardiovascular (CV) system, and respiratory systems (ICH S7A 2004; ICH S7B 2005). Supplemental tests on these "core battery" organ systems and on other organ systems are conducted on a case-by-case basis. The relevant pharmacodynamic endpoints are measured following a single, or sometimes multiple, dose administration of the pharmaceutical (depending on intended clinical dose schedule) and are most frequently conducted in rodents, with additional cardiovascular parameters being assessed in non-rodents and in vitro. The in vitro tests may include an evaluation of the ionic current through a native or heterologously expressed IKr channel protein, such as that encoded by hERG (the human Ether-à-go-go-Related Gene) and electrophysiological parameters in isolated cardiac preparations (ICH S7B 2005).

For anticancer therapies, there is a need to accelerate and streamline the development programs to ensure that patients receive the needed therapies expeditiously. This is especially the case for those agents being developed for late-stage disease in which the therapeutic options are limited. Because of the seriousness of many cancers, the potential benefit of efficacious therapeutics often outweighs the risk of producing adverse effects. Therefore, a certain level of toxicity may be tolerated in life threatening disorders that would be intolerable in other less severe indications. Consequently, the therapeutic index for many anticancer agents can be quite low. The attrition rate for anticancer drugs (as high as 95 %) is generally higher than for other drugs, primarily due to poor effectiveness at tolerable doses (Kola and Landis 2004). Therefore, because many molecules need to be tested in order to find the optimal molecule for cancer therapy, it is important that the

nonclinical programs are streamlined and accelerated while adequately addressing patient safety and minimizing the use of animals as per the 3 R's (replace, refine, reduce) (see Flecknell 2002). To that end, the ICH S9 guidance document for nonclinical evaluation for anticancer pharmaceuticals (ICH S9 2009) was developed. With regards to safety pharmacology, the ICH S9 guidance states that "an assessment of the pharmaceutical's effect on vital organ functions (including cardiovascular, respiratory, and central nervous systems) can be included in general toxicology studies." This implies that the conduct of separate dedicated safety pharmacology studies is not required (Cavero 2011). "Detailed clinical observations following dosing and appropriate electrocardiographic (ECG) measurements in non-rodents are generally considered sufficient" (ICH S9 2009).

The ICH S9 guidance does not apply to "pharmaceuticals intended for cancer prevention, treatment of symptoms or side effects of chemotherapeutics" (ICH S9 2009). Since the ICH S9 guidance states that it is applicable to late-stage cancer, it can be implied that for early-stage cancer therapeutics that the ICH M3 (R2), ICH S7A, and ICH S7B guidelines should be applied. However, since many anticancer agents are tested first in late-stage patients and have demonstrated safety and efficacy before going to early-stage patients, sufficient safety information may be available in patients such that additional animal safety pharmacology studies to support use in early-stage patients are not warranted.

2 Considerations for Safety Pharmacology Testing for Small Molecule Pharmaceuticals Versus Large-Molecule Biopharmaceuticals

The types of nonclinical safety studies conducted to support early- and late-stage clinical development will depend upon the type of molecule (large-molecule biopharmaceutical versus small molecule pharmaceutical), the pharmacology of the molecule, and the intended clinical use. Cancer therapeutics represent a very diverse group of molecules that includes, chemically defined small molecule drugs, naturally occurring toxins, cytokines, monoclonal antibodies (mAbs), and monoclonal antibody toxin/drug conjugates (ADCs). Most of the chemically synthesized pharmaceutical drugs and toxins have molecular weights of less than 1,000 Daltons (Da), whereas the protein biopharmaceuticals have molecular weights of greater than 10,000 Da. Small molecules are able to cross cellular and nuclear membranes and to gain access to intracellular structures and nuclear material. Molecules with a low molecular weight that are non-ionized, lipid soluble, and not plasma protein bound will readily diffuse across biological membranes (Ratain and Plunkett 2003). Therefore, small molecules that have the optimal properties for diffusion have the potential to act both extracellularly and intracellularly. For most of the small molecule anticancer pharmaceuticals, the primary site of action is within the cell, and their ability to traverse biological membranes is an advantage. However, pharmaceutical drugs are rarely specific for their intended pharmacological target and can be described as either selective or nonselective depending upon their

affinity for their intended target versus non-intended targets. In addition, many chemicals and drugs are transformed by hepatic enzymes into a variety of metabolites, some of which may be pharmacologically active or toxic. Consequently small molecules have the potential to produce "off-target" toxicities (not related to the primary mechanism of action) as well as "on-target" toxicities (related to the primary mechanism of action). These can be due to the parent molecule or the metabolites. Therefore, for many small molecules, an understanding of the primary pharmacology of the parent molecule alone may not be sufficient to determine whether there will be unintended toxicities in vivo. An understanding of potential sites of action, affinity for intended and unintended targets, biodistribution, and metabolism all play a role in the overall risk assessment.

The potential risks associated with the administration of biopharmaceuticals differ from that of traditional pharmaceutical drugs (Vugmeyster et al. 2012). Biopharmaceuticals are large molecular weight proteins that are manufactured using recombinant DNA technology. Monoclonal antibodies have molecular weights in the 150,000 Da range, and cytokines have molecular weights in the tens of thousands. Their large size alone precludes them from diffusing into cells, and therefore, they produce their pharmacological actions extracellularly either by interacting with cell surface receptors or by binding to circulating soluble factors. The monoclonal antibodies constitute the largest class of biopharmaceutical anticancer agents. These are mostly antagonist molecules that bind with a high degree of selectivity to cell surface receptors on tumors or to the receptor ligand and thereby inhibit pathway signaling (Scott et al. 2012). Cross reactive binding to unintended target antigens is extremely rare for antibodies. Cytokines on the other hand are potent agonist molecules that produce their pharmacological effects by activating cell surface receptors (Dranoff 2004). Although selective in terms of molecular targeting, cytokines are pleiotropic in that they can activate multiple cell types that express the target. Proteins that contain only naturally occurring amino acids are not metabolized by liver enzymes. Instead they undergo proteolysis to the component amino acids which are then reused by the body (Vugmeyster et al. 2012). Consequently, proteolysis of recombinant proteins does not generally result in the generation of toxic metabolites (Hamuro and Kishnani 2012). Overall, because of the highly selective pharmacology of biopharmaceuticals, and their restricted distribution in the body, off-target toxicities are rare. Therefore, an understanding of the primary pharmacology of the biopharmaceutical may be sufficient to determine the potential for inducing unwanted effects in the clinic. However, it may be the case that not all of the pharmacological effects are appreciated in early development, and an assessment of potential adverse effects in the nonclinical setting maybe warranted.

The conduct of dedicated safety pharmacology studies has not routinely been conducted for biopharmaceuticals, even for those agents that were developed prior to the ICH S9 guidance document. This is because the nonclinical safety testing of biopharmaceuticals differs from that of small molecule pharmaceuticals and is described in the ICH S6 (R1) guidance document (ICH S6 (R1) 2011). This guidance document emphasizes the need for a case-by-case approach to the

nonclinical safety testing of biopharmaceuticals and also stresses the need for the selection of a species that is pharmacologically relevant (ICH S6 (R1) 2011). A relevant species is defined as one in which the test material is pharmacologically active due to the expression of the receptor or an epitope (in the case of monoclonal antibodies). The ICH S6 (R1) and ICH S7A guidance documents provide the option for conducting safety pharmacology assessments as part of the general toxicity studies unless there is a specific concern. This is particularly important for biopharmaceuticals because many biopharmaceuticals are highly species restricted, and the only pharmacologically relevant species may be nonhuman primates (NHP). The 3Rs are particularly important when NHPs are being considered (Chapman et al. 2009; Buckley et al. 2011).

If the target exists only on tumors, then it may not be possible to identify a pharmacologically relevant animal species for testing of the biopharmaceutical. In this case, a single study in a rodent species could be used to test for potential off-target effects. This could be evaluated as part of the rodent tumor pharmacology studies. Alternatively a rationale could be proposed for initiating clinical studies based upon in vitro testing only. However, each of these strategies would require a strong rationale and agreement from the reviewing regulatory agencies. Without relevant animal safety information, the clinical starting dose and dose escalation scheme will likely need to be very conservative.

The in vitro hERG assay that is routinely conducted as part of the preclinical cardiovascular safety testing for small molecule pharmaceuticals is not appropriate for monoclonal antibodies because they have very low potential to interact with the extracellular or intracellular (pore) domains on the hERG channel (Vargas et al. 2008). In addition, their large size would preclude them from being able to access and block the inner pore of the hERG channel where many small molecule pharmaceuticals bind.

Clearly the type of molecule, its biodistribution, its mode of metabolism and potential generation of active or toxic metabolites, and the mechanism of action of the molecule are important factors in determining the appropriate studies that should be conducted to address safety pharmacology concerns.

3 Small Molecule Anticancer Agents

The earliest small molecule anticancer agents primarily targeted DNA and inhibited cell replication. Their actions were largely directed towards rapidly dividing cells. Rapidly dividing cancer cells are the intended target, but other rapidly dividing cells such as the bone marrow, the epithelial cells of the gastrointestinal tract, the liver, and the germ cells of the reproductive tract may also be affected (Colvin 2003). In addition, a variety of cardiac toxicities have been observed for these agents (Bovelli et al. 2010). More recently developed agents, e.g., tyrosine kinase inhibitors (TKIs) have been designed to be selective for specific types of cells regardless of rate of cell division, by targeting specific pathways that are activated in cancer cells, sparing normal cells. However, many of these tumor-selective agents have also been associated with cardiac toxicity.

The small molecule anticancer agents are too numerous to describe individually in this chapter, but some representative molecules or classes are listed in Tables 1 and 2, and a few examples of those that have been associated with cardiovascular toxicity or other relevant target organ toxicities are described below.

Table 1 Non-antibody anticancer agents associated with cardiovascular toxicities

Agent (M_{wt})	Clinical adverse findings
Cyclophosphamide (279 Da)	Acute HF with pericardial effusion, myocardial hemorrhagic necrosis, interstitial edema, fibrin deposits, microvascular thrombi, ischemia, ECG changes
Ifosfamide (261 Da)	LV dysfunction, arrhythmia, acute heart failure, pulmonary edema, ventricular tachycardia and fibrillation, ECG changes
Cisplatin (300 Da)	Hypertension, myocardial ischemia, acute coronary syndromes, LV dysfunction
Mitomycin C (334 Da)	Enhanced anthracycline cardiotoxicity
Busulphan (246 Da)	Pericardial and myocardial fibrosis, tachycardia, hypertension or hypotension, LV dysfunction
5-Fluorouracil (130 Da)	Myocardial ischemia, acute heart failure, hypotension, electrocardiographic changes, acute coronary syndromes
Capecitabine (359 Da)	Myocardial ischemia, ventricular arrhythmias
Gemcitabine (299 Da)	Pericardial effusion, thromoembolic events, vasculitis
Fludarabine (285 Da)	Hypotension, heart failure
Cytosine arabinoside (242 Da)	Pericarditis, pericardial effusion, cardiac tamponade
Taxanes: Paclitaxel (853 Da) Docetaxel (861 Da)	Sinus bradycardia, ventricular arrhythmias, myocardial ischemia, LV dysfunction, enhanced anthracycline cardiotoxicity
Vinca alkaloids: Vincristine (923 Da) Vinblastine (909 Da) Vinorelbine (778 Da)	Autonomic neuropathy (peripheral neuropathy)
Etoposide (588 Da)	Hypotension
Bleomycin (1,415 Da)	Pulmonary fibrosis, pericarditis
Estramustine (582 Da)	Thromboembolic events
Retinoic acid (300 Da)	Hypotension, pleural and pericardial effusion, LV dysfunction, acute renal failure
Arsenic trioxide (197 Da)	QT interval prolongation, pleural and pericardial effusions
Thalidomide (258 Da)	Sinus bradycardia, edema, deep vein thrombosis
Interferon alpha-2b (~19,000 Da)	Hypotension, arrhythmia, or tachycardia
Interferon gamma-1b (~17,000 Da)	Exacerbation of preexisting cardiac conditions (ischemia, CHF, arrhythmias)
Interleukin-2 (~15,000 Da)	Capillary leak syndrome, hypotension, arrhythmias

For reviews, refer to Monsuez et al. (2011) and Senkus and Jassem (2011)

Table 2 Effects identified from animal safety pharmacology and general toxicity studies, and clinical adverse effects observed in cancer patients for approved tyrosine kinase inhibitors

Agent: Target: the US Approval Date	Safety pharmacology studies in animals or in vitro (systems evaluated: findings)	Safety pharmacology in animal general toxicity studies[a]	Target organs (excluding reproductive organs) toxicities in animal general toxicity studies[a]	Adverse findings in patients[b]
Imatinib (Gleevec/Glivec): Bcr-Abl, PDGF, SCF, c-kit: 2001	CV, pulmonary, CNS, renal, GI: Mild sedative effects, slight transient↑BP	ECG in monkey—no effect	6-month rat, 9-month monkey Bone marrow, liver, GI	CHF, LV dysfunction, fluid retention, hemorrhage, GI perforations, cytopenias, hepatotoxicity, bullous dermatologic reactions, hypothyroidism, growth retardation in children with tumor lysis syndrome (TLS)
Gefitinib (Iressa): EGFR, VEGF, PDGF, FGF, IGF: 2003 (discontinued)	CV, CNS, pulmonary, renal, GI: ↓hERG current, ↑APD, QT prolongation, ↓BP, ↑HR, antidiuretic, ↓pulmonary function	ECG in dog—↑PR interval	6-month rat, 6-month dog Dermatologic, GI, corneal atrophy, liver and kidney necrosis	Skin disorders, diarrhea, interstitial lung disease (ILD), hepatotoxicity, GI perforation, conjunctivitis, hemorrhage
Erlotinib (Tarceva): EGFR: 2004	CV, CNS, pulmonary, renal, GI: ↓gastric emptying	ECG in dog—no effect	6-month rat, 12-month dog Liver, dermatologic, and ophthalmic	MI, bleeding events, microangiopathic hemolytic anemia, ILD, acute renal failure, hepatotoxicity, GI perforations, corneal perforation and ulceration, bullous, and exfoliative skin disorders

(continued)

Table 2 (continued)

Agent: Target: the US Approval Date	Safety pharmacology studies in animals or in vitro (systems evaluated: findings)	Safety pharmacology in animal general toxicity studies[a]	Target organs (excluding reproductive organs) toxicities in animal general toxicity studies[a]	Adverse findings in patients[b]
Sorafenib (Nexavar): c-RAF, B-RAF, mutant B-RAF, c-KIT, FLT-3, RET, VEGFR-1, 2, 3, PDGFR-ß: 2005	CV, CNS, pulmonary, renal, GI: Antidiuretic, hypoglycemic, anti-nociceptive, tremor, ↑GI motility, ↓HR	ECG in dog—no effect	6-month rat, 12-month dog Bone growth plate, kidney, liver, spleen, GI, bone marrow	QT Prolongation, hypertension, MI, bleeding, hand–foot skin reaction and rash, GI perforation
Sunitinib (Sutent): PDGFRα, β, VEGFR-1, 2, 3, KIT, FLT3, CSF-1R, RET 2006	CV, CNS, pulmonary: QT prolongation, ↑ tidal volume	ECG and ECHO in monkey—↓ HR, ↓LVEF	6-month rat, 9-month monkey Bone growth plate, kidney, pancreas, bone marrow, liver	QT prolongation and Torsade de Pointes, ↓LVEF, hypertension, hemorrhagic events, hepatotoxicity, osteonecrosis of the jaw, TLS, thyroid dysfunction (hypothyroidism or hyperthyroidism)
Dasatinib (Sprycel): Bcr-Abl, SRC, LCK, YES, FYN, c-KIT, EPHA2, and PDGFRβ: 2006	CV: ↑BP, ↓hERG current, ↑APD	ECG in monkey—no effect	6-month rat, 9-month monkey: GI, kidney, heart, liver, lymphoid/hematopoietic, adrenals, thyroid, pancreas, lung	QT Prolongation, CHF, LV dysfunction, MI, hemorrhage, Pulmonary Arterial Hypertension (PAH), fluid retention, myelosuppression
Lapatinib (Tykerb): HER2, EGFR: 2007	CV, CNS, pulmonary: occasional premature ventricular contractions	ECG in dog—no effect	6-month rat, 9-month dog: Skin, GI, liver, lymphoid, adrenal	QT prolongation, ↓LVEF, interstitial lung disease and pneumonitis, diarrhea, hepatotoxicity

Nilotinib (Tasigna): Bcr-Abl, PDGFR, c-KIT, CSF-1R, DDR1: 2007	CV, CNS, respiratory: ↓hERG current, ↓ coronary flow	ECG in monkey—no effect	6-month rat, 9-month monkey: Liver, kidney, lung, heart, spleen	QT Prolongation, hepatotoxicity, elevated serum lipase, electrolyte abnormalities, TLS, myelosuppression
Pazopanib (Votrient): VEGFR-1, 2, 3, PDGFR-α, β, FGFR 1, 3, c-Kit, ITK, LCK, c-Fms: 2009	CV, neurological, pulmonary. ↑BP, ↓HR	ECG in monkey—no effect	6-month rat, 12-month monkey: Bone plate changes, broken teeth (rats), kidney, trachea, adrenal, pituitary, lymph node	QT interval prolongation CHF, ↓ LVEF and ↓ LVEF, hypertension Hemorrhagic events, arterial and venous thrombotic events, GI perforation or fistula, Reversible Posterior Leukoencephalopathy Syndrome (RPLS), hepatotoxicity, hypothyroidism, proteinuria, serious infections
Crizotinib (Xalkori): ALK, HGFR (c-met), RON: 2011	CV, CNS, pulmonary: ↓hERG conductance, ↓HR, ↓LVEDP, ↓LV + dP/dt, ↑PR, QRS, and QT intervals, ↓locomotor activity, ↓RR	ECG dog—↑QT interval	3-month rat, 3-month dog: Bone marrow, GI, heart, liver, lung, lymph node, pituitary, salivary gland	QT Interval prolongation, hepatotoxicity, pneumonitis
Vandetanib (Caprelsa): EGFR, VEGFRs, RET, BRK, TIE2, EPHR, Src: 2011	CV, neurological, pulmonary, renal, GI: ↓hERG conductance, ↑ AP duration, ↑QT, ↑ BP, proteinuria, ↓ GI motility	ECG dog—↑QT interval	6-month rat, 9-month dog: Adrenal gland, bile duct, heart, kidney, lungs, pancreas, lymph node, skin, spleen, stomach, teeth, and thymus	QT prolongation and Torsade de pointes, skin, interstitial lung disease, ischemic cerebrovascular events, hemorrhage, HF, GI, hypothyroidism, hypertension, RPLS

(continued)

Table 2 (continued)

Agent: Target: the US Approval Date	Safety pharmacology studies in animals or in vitro (systems evaluated: findings)	Safety pharmacology in animal general toxicity studies[a]	Target organs (excluding reproductive organs) toxicities in animal general toxicity studies[a]	Adverse findings in patients[b]
Axitinib (Inlyta): VEGFR-1, 2, and 3: 2012	CV, CNS, pulmonary, GI ↑ gastric emptying ↑BP, ↓HR	ECG in dog—no effect	6-month mouse, 9-month dog: Broken teeth (mice), spleen	Hypertension, arterial and venous thrombotic events, hemorrhagic events, gastrointestinal perforation, hypothyroidism, RPLS, proteinuria, liver enzyme elevation

ALK, Anaplastic lymphoma kinase; c-Fms, transmembrane glycoprotein receptor tyrosine kinase, c-Kit; stem cell factor (CD117); CSF, colony stimulating factor; EGFR, epidermal growth factor receptor; FGFR, fibroblast growth factor receptor; FLT, Fms-like tyrosine kinase; HGFR, hepatocyte growth factor receptor; ITK, interleukin-2 receptor inducible T-cell kinase; LCK, leukocyte-specific protein tyrosine kinase; PDGFR, platelet-derived growth factor receptor; RET, glial cell line-derived neurotrophic factor; receptor RON, Recepteur d'Origine Nantais; CV, Cardiovascular; CNS, Central Nervous System; GI, gastrointestinal; BP, blood pressure; HR, heart rate; RR, respiratory rate; APD, Action Potential Duration; LVEDP, Left Ventricular Diastolic Pressure
Note: Effects on the reproductive system have not been included in this analysis
[a]Nonclinical information for each of the compounds listed can be found in the US FDA Pharmacology/Toxicology Reviews at http://www.accessdata.fda.gov or in the European Public Assessment Reports (EPAR) at http://www.ema.europa.eu/ema
[b]The clinical information can be found on the prescribing information for each of the individual products

3.1 Anthracyclines

Anthracyclines and related compounds are intercalating agents originally derived from *Streptomyces* bacteria. The anthracyclines comprise of doxorubicin, daunorubicin, zorubicin, epirubicin, and idarubicin. Other related molecules include mitoxantrone, bisantrene, aclacinomycin A, and amsacrine. Although highly efficacious against a number of cancers (such as leukemia, lymphoma, and solid tumors), these drugs are associated with cumulative dose-dependent cardiac toxicity (Ferreira et al. 2008; Monsuez et al. 2011). They cross the cellular and nuclear membranes and integrate into nuclear DNA where they inhibit transcription and protein synthesis, inhibit topoisomerase II, and thereby inhibit DNA repair. These compounds also generate reactive oxygen species (such as hydrogen peroxide and hydroxyl radicals) producing further DNA damage and inducing apoptosis of cells (Berthiaume and Wallace 2007; Zhang et al. 2009). Doxorubicin produces both acute cardiac toxicities as well as cumulative cardiac toxicities. The acute effects consist of aberrant ECG changes such as ST-T-wave alterations, atrial fibrillation, acute heart failure, myocarditis, and myocardial infarction in ~1 % of patients. The cumulative effects consist of diastolic dysfunction, followed by systolic dysfunction eventually leading to symptomatic heart failure that is unresponsive to digitalis. Increases in serum troponin I levels (TnI) have been used as an indicator for patient stratification of anthracycline-induced cardiac toxicity (Cardinale et al. 2004, 2010).

The cardiac toxicity of anthracyclines can be reproduced in numerous animal species (Working et al. 1999). Histopathologically, the cardiac effects consist of damage to the sarcoplasmic reticulum (due to drug interaction with ryanodine receptor-mediated calcium-induced calcium release), and, as a consequence, calcium overload results in mitochondrial swelling and altered outer membrane permeability that can occur within hours of administration and progress with repeated exposures. The myocardial damage can also be induced in vitro in isolated heart tissues (Menna et al. 2007; Robert 2007).

To improve antitumor efficacy while reducing toxicity, encapsulation of anthracyclines in liposomal preparations has been developed (DaunoXome, Doxil/Caelyx, and Myocet/Evacet) (Working et al. 1999; Ewer et al. 2004; Rahman et al. 2007).

3.2 Tyrosine Kinase Inhibitors

Tyrosine Kinases (TKs) are a family of intracellular enzymes that catalyze the transfer of phosphate residues from ATP to tyrosine residues in intracellular proteins. TKIs play a key role in the transmission of signals for cell growth, differentiation, migration, apoptosis, and in angiogenesis. TKIs can either be associated with a membrane receptor that is activated by extracellular ligands such as growth factors or cytokines or can be non-membrane bound in which case they transmit signals within the cell. Dysregulation of tyrosine kinases have

been implicated in malignant transformation of cells (Krause and Van Etten 2005). This signaling can persist even in the absence of activating signals. Although the TKIs were designed to specifically target cancer cells, recent clinical data has indicated that some of these agents may be associated with cardiac toxicity including left ventricular dysfunction (i.e., reduced left ventricular ejection fraction and QT prolongation) and congestive heart failure (Mellor et al. 2011; Table 2).

3.2.1 Imatinib

The first approved small molecule TKI for the treatment of cancer was imatinib (Gleevec) in 2001 in the US (and Glivec (2004) in the EU). Imatinib was originally described as an inhibitor of the Bcr-Abl tyrosine kinase but has subsequently been shown to inhibit other tyrosine kinases: Kit, the receptor for stem cell factor (SCF) coded for by the c-Kit proto-oncogene, the discoidin domain receptors (DDR1 and DDR2), the colony stimulating factor receptor (CSF-1R), and the platelet-derived growth factor receptors alpha and beta (PDGFR-alpha and PDGFR-beta). Clinical use of imatinib has been associated with left ventricular dysfunction (i.e., reduced left ventricular ejection fraction) and congestive heart failure (Gleevec Prescribing Information (PI), 2013).

The nonclinical safety program to support the clinical use of imatinib consisted of safety pharmacology studies in rat (CV, pulmonary, renal, GI), mouse (CNS), and dog (pulmonary and CV) (Gleevec Pharmacology Review US FDA 2001; Glivec European Public Assessment Report (EPAR) 2004). The only finding in the safety pharmacology studies was a transient reduction in arterial blood pressure in rats. Imatinib was shown not to be genotoxic. Repeated-dose toxicology studies were conducted in rats (5–50 mg/kg/day) for up to 6-months, and in monkeys (15–80 mg/kg/day), for up to 9-months. In the toxicology studies, the main target organs of toxicity consisted of bone marrow, lymphoid tissues, testis/ovaries, and gastrointestinal tract (Glivec EPAR, 2004). Cardiac toxicity was not identified from standard histopathological examination of the hearts.

Although the toxicology and safety pharmacology studies conducted with imatinib in normal rats and monkeys failed to identify the cardiac toxicities seen in patients, subsequent exploratory studies conducted in mice and rats were able to reveal cardiac abnormalities similar to those seen in patients (Kerkelä et al. 2006; Herman et al. 2011). In mice dosed with imatinib (5–200 mg/kg/day) for 3 or 6 weeks, LV function (measured by echocardiography) was impaired, and electronmicroscopic examination of the heart revealed mitochondria sarcoplasmic reticulum abnormalities. In vitro studies showed that imatinib produced direct toxicity to cardiomyocytes and that this toxicity was directly related to c-Abl inhibition (Kerkelä et al. 2006). In normal and hypertensive rats, repeated dosing of imatinib (10–100 mg/kg) produced a complex pattern of cellular alteration that resulted in cardiotoxicity that was influenced by hemodynamic status (Herman et al. 2011). Hasinoff and Patel (2010) examined 18 TKIs and suggest that the cardiotoxicity observed may actually result from a *lack* of specific binding to both tyrosine kinases and serine-threonine kinases rather than either kinase specifically. Thus the cardiotoxicity induced by imatinib, although unanticipated based upon the

toxicology studies conducted in normal animals, does appear to be directly related to its mechanism of action, i.e., kinase inhibition induced myocyte damage.

3.2.2 Sunitinib

Sunitinib (Sutent) is an example of a multi-targeted TKI. It inhibits PDGFRα and PDGFRβ, vascular endothelial growth factor receptors (VEGFR1, VEGFR2, and VEGFR3), KIT, Fms-like tyrosine kinase-3 (FLT3), CSF-1R, and the glial cell-linederived neurotrophic factor receptor (RET) (Mena et al. 2010). In patients, cardiac failure, cardiomyopathy, pericardial effusion, left ventricular failure, and QT prolongation are described as uncommon adverse events (0.1–0.4 %), whereas hypertension is described as a very common event (23 %) (Sutent PI 2012; Chu et al., 2011).

The nonclinical safety studies conducted to support the clinical use of sunitinib included core battery safety pharmacology studies (cardiovascular, CNS, respiratory) and general toxicology studies (Sutent EPAR 2006; Sutent Pharmacology Review US FDA 2006). In in vitro cardiovascular safety pharmacology studies, sunitinib inhibited IKr channel conduction ($IC_{50} = 266nM$) and increased the cardiac action potential duration (APD_{90}) in canine Purkinje fiber cells. In vivo, sunitinib has been shown to increase arterial blood pressure (Blasi et al. 2012) and prolong the QT interval. No toxicologically significant effects on heart rate, body temperature, or locomotor activity were noted. In rats, there were no effects on general behavior, body temperature, or respiratory parameters.

The repeat-dose toxicology studies were performed in rats and monkeys (Sutent EPAR 2006; Sutent Pharmacology Review US FDA 2006; Patyna et al. 2008). Target organs of toxicity included the hematopoietic organs (thymus, bone marrow, spleen, and lymph nodes), gastrointestinal tract, glands (adrenal, pancreas, and salivary), bone growth plates, and female reproductive organs. The effects on the bone growth plates (inhibition of bone plate angiogenesis leading to physeal dysplasia and skeletal abnormalities) and on the reproductive organs (decreased angiogenesis in the ovarian follicles) are characteristic effects of VEGF inhibition (see also bevacizumab). In the repeat-dose toxicology studies performed in monkeys, treatment caused a modest decrease in heart rate, and in the 9-month study in which echocardiography was conducted, a decrease in cardiac preload and contractility was noted. In an exploratory study in monkeys, QT prolongation and compromised ventricular function were observed (Sutent EPAR 2006; Sutent Pharmacology Review US FDA 2006).

Additional exploratory studies designed to obtain a better understanding of the clinically observed cardiac toxicities were able to identify abnormalities of cardiomyocytes with mitochondrial swelling and degenerative changes in sunitinib-treated mice (Chu et al. 2007). In normotensive mice, sunitinib did not induce cardiomyocyte apoptosis or increase in blood pressure similar to that seen in patients, but when blood pressure was increased by the administration of an α-adrenergic agonist, cardiomyocyte apoptosis was observed, suggesting that left ventricular dysfunction might be due, in part, to direct cardiomyocyte toxicity, exacerbated by hypertension (Chu et al. 2007).

4 Large-Molecule Anticancer Agents

The large-molecule anticancer agents largely consist of cytokines, monoclonal antibodies (mAbs), and antibody-drug conjugates (ADCs) (Tables 1 and 2).

The cytokines (interferon (IFN)-α,-IFN-γ, and interleukin (IL)-2) are associated with toxicities attributable to their potent stimulation of the immune system (Dranoff 2004). The most frequently reported toxicities ("flu-like symptoms," i.e., fever, headache, chills, myalgia, and fatigue, Intron-A PI 2012; Actimmune PI 2009; Proleukin PI 2012) are similar to those that might be observed during a viral infection. However, with the doses used to treat cancer, a variety of cardiovascular toxicities have also been reported (Table 2).

Unconjugated monoclonal antibodies generally have less toxicity than cytokine therapeutics and many of the other types of anticancer therapeutics (Table 3). The monoclonal antibodies that are currently approved for cancer either inhibit the binding of growth factors such as vascular endothelial growth factor (VEGF) or epidermal growth factor (EGF) to their receptors, and/or they bind to their target on tumor cells (e.g., HER2, CD20, CD52) and induce target cell killing through antibody-dependent cell cytotoxicity (ADCC; Weiner 2007). Ipilimumab (Yervoy), which has recently been approved for the treatment of non-resectable metastatic melanoma, differs from the other approved anticancer monoclonal antibodies in that it blocks an inhibitory pathway (CTLA-4) resulting in T-cell activation and a number of associated toxicities (Graziani et al. 2012; Yervoy PI 2012). In general, the toxicities associated with monoclonal antibody administration are either directly related to their primary mechanism of action or are the result of nonspecific immunologically mediated effects such as infusion reactions (characterized by fever and chills, and occasional nausea, pain, especially at tumor sites, headache, hypotension, and rash).

Antibody-drug/toxin conjugates (ADCs) combine the selective tumor targeting properties of monoclonal antibodies to direct a toxin (radiation or chemical toxin) specifically to the tumor cells (Govindan and Goldenberg 2010). The goal of this approach is that by targeting the toxin to the tumors, efficacy will be enhanced, while off-target toxicity is reduced. Currently the only FDA-approved ADC is Brentuximab vedotin (Adcetris), which is used to treat anaplastic large cell lymphoma (ALCL) and Hodgkin lymphoma (Adcetris IP 2012). This monoclonal antibody targets CD30, a cell membrane protein of the tumor necrosis factor receptor family. As might be expected, the toxicity of this conjugate is primarily driven by the toxicity of the toxin [monomethyl auristatin E (MMAE)] moiety (Table 3). Because ADCs combine an antibody with a small molecule drug, the nonclinical safety program needs to be a hybrid between that described in ICH S6 (R1) for biopharmaceuticals and that described in ICH M3 (R2) for pharmaceutical drugs (Roberts et al. 2013).

Table 3 Effects identified from animal safety pharmacology and general toxicity studies, and clinical adverse effects observed in cancer patients for approved monoclonal antibody anticancer therapeutics

Agent: Target: US Approval Date	Safety pharmacology studies in animals or in vitro (systems evaluated: findings)[a]	Safety pharmacology in animal general toxicity studies[a]	Target organs toxicities in animal general toxicity studies[a]	Adverse findings in patients[b]
Rituximab (Rituxan/ MabThera): CD20: 1997	Not conducted	No information	2-month monkey: B-cell depletion	Tumor lysis syndrome (TLS), progressive multifocal leukoencephalopathy (PML), hepatitis B, infections, cardiac arrhythmias and angina, bowel obstruction and perforation, cytopenias
Trastuzumab (Herceptin): HER2: 1998	ECG monkey PK studies: no effect	No information	6-month monkey: No toxicity	Cardiomyopathy, infusion reactions, pulmonary toxicity, exacerbation of chemotherapy-induced neutropenia
Alemtuzumab (Campath): CD52: 2001	CV, pulmonary in monkeys: ↓BP, ↑HR	No information	1-month monkey (MAHA response precluded further dosing): Lymphocytopenia	Cytopenias, Infections
Bevacizumab (Avastin): VEGF: 2004	Not conducted	ECG in monkey—no effect	6-month monkey: Bone plate changes	Non-gastrointestinal fistula formation, arterial thromboembolic events (e.g., myocardial infarction, cerebral infarction), hypertension, reversible posterior leukoencephalopathy syndrome (RPLS), proteinuria, infusion reactions

(continued)

Table 3 (continued)

Agent: Target: US Approval Date	Safety pharmacology studies in animals or in vitro (systems evaluated: findings)[a]	Safety pharmacology in animal general toxicity studies[a]	Target organs toxicities in animal general toxicity studies[a]	Adverse findings in patients[b]
Cetuximab (Erbitux): EGFR: 2004	CV, pulmonary in monkeys: ↓BP, ↑HR, ↑ RR rate, ↓ tidal volume	ECG in monkey—no effect	9-month monkey: Dermatologic toxicity and secondary infections	Infusion reactions, cardiopulmonary arrest, pulmonary toxicity, dermatologic toxicity, hypomagnesemia
Panitumumab (Vectibix): EGFR: 2006	CV, CNS, pulmonary in monkey: No effects	ECG in monkey—no effect	6-month monkey: Dermatologic toxicity, infusion reactions, GI	Dermatologic toxicity, infusion reactions, pulmonary fibrosis/ interstitial lung disease (ILD): Electrolyte Depletion, ocular toxicities
Ofatumumab (Arzerra): CD20: 2009	Not conducted	ECG in monkey—no effect, clinical observations of ↑HR and force and tremors	7-month monkey: Infusion reactions, B-cell depletion, infection, anemia	Infusion reactions, cytopenias, PML, hepatitis B infection, and reactivation
Ipilimumab (Yervoy): CTLA-4: 2011	Not conducted	Not conducted	6-month monkey: No organ toxicity ↑ antibody response to viral vaccine challenge	Immune-mediated adverse reactions due to T-cell activation and proliferation. Immune-mediated hepatitis, immune-mediated endocrinopathies:

brentuximab vedotin (Adcetris): CD30-MMAE: 2011	hERG CV, pulmonary, CNS monkeys: No effects	ECG, HR, BP, RR in monkey—no effect	1-month rat, 6-month monkey: ↓WBC, ↓RBCs, ↑liver enzymes (rats only)	Peripheral neuropathy, infusion reactions, neutropenia, TLS, PML, Stevens–Johnson syndrome (skin)

Note: Effects on the reproductive system have not been included in this analysis. *MAHA* monkey anti-human antibody

[a]Nonclinical information for each of the compounds listed can be found in the US FDA Pharmacology/Toxicology Reviews at http://www.accessdata.fda.gov or in the European Public Assessment Reports (EPAR) at http://www.ema.europa.eu/ema

[b]The clinical information can be found on the prescribing information for each of the individual products

4.1 Monoclonal Antibodies

4.1.1 Trastuzumab

Trastuzumab (Herceptin) is a humanized monoclonal antibody against the extracellular domain of the human epidermal growth factor receptor 2 (HER2). Treatment of cancer patients with trastuzumab has been associated with an increased incidence of cardiac toxicity including left ventricular cardiac dysfunction, arrhythmias, hypertension, disabling cardiac failure, cardiomyopathy, and cardiac death. Trastuzumab can also cause asymptomatic decline in left ventricular ejection fraction (LVEF) (Herceptin PI 2010).

In patients receiving trastuzumab as either a single agent or in combination therapy, when compared with those not receiving trastuzumab, there was a ~4–6-fold increase in the incidence of symptomatic myocardial dysfunction. The highest incidence occurs when trastuzumab is administered concurrently with an anthracycline. One possible reason for the cardiac toxicity with trastuzumab is that HER2 is not only expressed on breast cancer tumors but is also expressed on cardiomyocytes, albeit at lower levels (Negro et al. 2004). The HER2 signaling pathways in the heart appear to be important for cardiac myocyte development, survival, and adaptation to stress (Zhao et al. 1998). In contrast to anthracycline-induced cardiac toxicity, trastuzumab-related cardiac dysfunction does not increase with dose, is not associated with ultrastructural changes in the myocardium, and is generally reversible. When used in the adjuvant treatment of ErbB2-positive early-stage breast cancer, trastuzumab significantly improves survival. The incidence of class III/IV congestive heart failure (CHF) has been shown to range from 0.4 % to 3.8 % in trastuzumab trials (see Perez 2008). Note that cardiac HER2 expression is upregulated following doxorubicin treatment. One hypothesis for the observed cardiotoxicity is that anthracyclines cause myocyte oxidative stress and necrosis, and when trastuzumab is administered, the normal cellular repair mechanism is inhibited, thus enhancing the toxicity.

Cardiac toxicity was not predicted from the nonclinical safety studies (Herceptin EPAR 2004). Trastuzumab binds only to human and monkey HER2; therefore, the nonclinical safety studies were limited to studies in monkeys. Trastuzumab was tested in a 4-week study in rhesus macaques and in 12- and 26-week studies in Cynomolgus macaques. No cardiac toxicity was identified in these studies. In single-dose combination pharmacokinetic studies, administration of trastuzumab together with paclitaxel, doxorubicin, or cyclophosphamide/doxorubicin in rhesus monkeys did not alter the pharmacokinetics of the other anticancer agents and did not produce any adverse effects on clinical observations, body weights, electrocardiograms, or clinical pathology.

Although the toxicology studies conducted in normal animals did not identify cardiotoxicity, studies conducted in genetically modified mice did reveal a potential risk of pathway inhibition (Crone et al. 2002). Mice that were generated to have a conditional ventricular-restricted deletion of ErbB2 (Her2/neu) developed dilated cardiomyopathy, including chamber dilation, wall thinning, and decreased contractility. Additionally, cardiomyocytes isolated from these conditional mutants were

more susceptible to anthracycline toxicity. ErbB2 signaling in cardiomyocytes is therefore important for the prevention of dilated cardiomyopathy.

4.1.2 Bevacizumab

Bevacizumab (Avastin) is a humanized recombinant IgG1 monoclonal antibody that binds to Vascular Endothelial Growth Factor (VEGF) and inhibits the binding of VEGF to its receptors, Flt-1 (VEGFR-1) and KDR (VEGFR-2), on the surface of endothelial cells. Inhibition of VEGF binding and signaling inhibits tumor angiogenesis, thereby inhibiting tumor growth. Bevacizumab is the first angiogenesis inhibitor to receive marketing authorization in the US to treat various cancers including metastatic colorectal and renal cell carcinoma, non-squamous non-small cell lung cancer, and glioblastoma multiforme of the brain (Avastin PI 2013).

Clinically, hypertension is a common (up to 34 %) cardiovascular event associated with bevacizumab treatment (Avastin PI 2013). Other cardiovascular events that may be associated with bevacizumab treatment include congestive cardiac failure, supraventricular tachycardia, and arterial thromboembolic events including cerebral infarction, transient ischemic attacks, myocardial infarction, angina, and hemorrhage (Nazer et al. 2011). The congestive heart failure may, in some cases, be associated with prior or concomitant use of anthacyclines.

Bevacizumab binds to and neutralizes human, monkey, and to a lesser extent rabbit VEGF but does not bind to rodent VEGF (Avastin Toxicology Data US FDA 2004; Avastin EPAR 2006). Therefore, monkey and rabbit are pharmacologically relevant species for the nonclinical safety assessment of bevacizumab. Repeated-dose toxicology studies in Cynomolgus monkeys for up to 26 weeks showed dose-related effects on sites of active neo-angiogenesis consisting of skeletal growth plate dysplasia, decreased ovarian and uterine weights, and an absence of corpora lutea (Ryan et al. 1999; Avastin Toxicology Data US FDA 2004). These effects are considered to be directly related to the primary mechanism of action of bevacizumab. No treatment-related effects were reported on physical examinations, including respiration rate, blood pressure, ECG, body temperature, and urinalysis. There were no histopathological changes in the cardiovascular tissues. Because of the mechanism of action of bevacizumab (inhibition of angiogenesis), exploratory studies were designed to test the potential of bevacizumab to inhibit wound healing in rabbits and monkeys. The studies conducted in rabbits showed a delay in wound healing, whereas the studies in monkeys were equivocal. To investigate the potential for bevacizumab treatment to increase the incidence of thrombosis, an acute rabbit model of thrombosis was used. This model showed no bevacizumab treatment-related effects (Avastin Toxicology Data US FDA, 2004).

4.1.3 Cetuximab and Panitumumab

Epidermal growth factor receptor (EGFR) is a transmembrane glycoprotein that is a member of a subfamily of type I receptor tyrosine kinases. Two recombinant monoclonal antibodies are currently marketed that bind to human EGFR and prevent the binding and signaling of EGFR ligands (EGF and TGFα). Cetuximab, because it is an IgG1 antibody, also binds to Fcγ receptors on effector cells

(monocytes and NK cells) and lyses EGFR expressing tumor cells through ADCC, whereas panitumumab which is an IgG2 antibody does not bind to Fcγ receptors and does not induce ADCC (Patel et al. 2010). Both are approved for use in the treatment of KRAS mutation negative (wild-type), EGF-expressing metastatic colorectal cancer, while cetuximab is also approved for use in head and neck cancer (Erbitux PI 2012; Vectibix PI 2012).

Cetuximab binds to human and monkey EGFR but does not bind to rodent EGFR. Therefore, the cynomolgus monkey was selected as a pharmacologically relevant species for nonclinical safety assessments (Erbitux Pharmacology Review US FDA 2004; Erbitux EPAR 2006). For cetuximab, a dedicated safety pharmacology study was conducted in Cynomolgus monkeys. This study showed no cetuximab treatment-related effects on heart rate, blood pressure, ECG, or respiratory rate. Safety pharmacology end points incorporated into the 9-month repeated-dose toxicology study also revealed no treatment-related effects. The major toxicity associated with cetuximab treatment was a skin toxicity which is directly related to the primary mechanism of action of EGFR inhibition. Diarrhea is also common in cetuximab-treated monkeys.

In patients treated with cetuximab, skin (predominantly an acne-like rash) and infusion (anaphylactic) reactions are commonly observed (Erbitux PI 2012). The infusion reactions can be associated with dyspnea, bronchospasm, and a reduction in blood pressure. Some rare cases of cardiopulmonary arrest have been reported with cetuximab in combination with radiation therapy.

For panitumumab, a dedicated safety pharmacology study was conducted in monkeys (Vectibix Pharmacology Review US FDA 2006; Vectibix EPAR 2007). In this study, panitumumab produced no treatment-related effects on the cardiovascular, respiratory, and central nervous systems. In a 1-month toxicology study in monkeys, some cardiac toxicity was observed. However, this was subsequently shown to be due to electrolyte imbalance due to severe diarrhea (likely related to inhibition of EGF) and did not occur in subsequent studies in which supportive fluids were administered. In the 6-month repeated-dose toxicology study, the major toxicity was skin toxicity similar to that described for cetuximab.

In the clinic, the most frequent adverse event associated with panitumumab is a dermatologic toxicity (Vectibix PI 2012). Infusion reactions also occur with panitumumab, and some rare cases of pulmonary fibrosis and embolism have been reported.

5 Discussion

Cardiovascular toxicities have been observed in patients with a number of anticancer agents. These toxicities have included hypertension, cardiomyopathy, left ventricular dysfunction, congestive heart failure, infarction, and thrombosis. In most cases, the standard repeated-dose toxicology studies conducted in normal animals have failed to predict these clinical toxicities. One reason may be that other comorbidities need to be present before the treatment-related cardiovascular

toxicities are manifested. The dedicated cardiovascular safety pharmacology studies that are designed to detect drug-induced conduction defects are not able to detect these types of functional defects but have been able to predict risk of inducting QT prolongation and subsequent arrhythmias. Although QT prolongation has been observed for a number of small molecule anticancer drugs, none of the monoclonal antibodies have shown QT prolongation, thus supporting the hypothesis that this liability is not a concern for monoclonal antibodies (Vargas et al. 2008).

In recent years, significant progress has been made in developing new anticancer therapeutics that are specifically designed to target cancer cells with the aim of increasing antitumor efficacy, while minimizing toxicity to non-cancer cells. These targeted agents include the small molecule tyrosine kinase inhibitors as well as monoclonal antibodies. Although these agents have fewer side effects in general than the nonselective cell replication inhibitors, unanticipated toxicities have been observed with some of these newer agents. Although most of these toxicities are manageable in cancer patients, there is still a need to further understand the mechanisms and to continue to improve the safety profile of anticancer agents.

Systolic dysfunction and heart failure are some of the most common cardiovascular side effects of tyrosine kinase inhibitors. This may occur because the pathways that are involved in the survival of cancer cells also appear to be involved in the survival of some normal cells (Chen et al. 2011). The tyrosine kinase inhibitors include molecules that target one or a few tyrosine kinases to those that target multiple tyrosine kinases. Although targeting multiple kinases may add efficacy, this also increases the likelihood for inducing toxicities. The toxicities that are observed appear to be on-target, i.e., directly related to the type of tyrosine kinase that is inhibited.

Inhibitors of the receptor tyrosine kinase HER2 (trastuzumab, lapitinib) and the non-receptor tyrosine kinase Bcr-Abl (imatinib, dasatinib) have been associated with cardiac toxicity. These effects appear to be directly related to the pharmacology of these kinases which play a protective role in the heart. The cardiac toxicity of these agents has generally not been detected in safety pharmacology studies or general toxicity studies in normal animals but has been demonstrated in specifically designed pharmacology studies in which the heart is subjected to concomitant cardiac stressors.

Inhibitors of VEGF signaling (bevacizumab, sorafenib, sunatinib, pazopanib, vandetanib, axitanib) inhibit tumor angiogenesis, thereby starving tumors and inhibiting their growth. Clinically, hypertension is consistently observed with the VEGF inhibitors. The mechanism for this increase in blood pressure in patients is not clearly understood, although it has been hypothesized that it may be due to an alteration in the release of endothelial-derived factors and to an effect on kidney function (Granger 2009). Proteinuria is also a common finding in patients treated with VEGF inhibitors. In general, the safety pharmacology studies and toxicology studies conducted in normal animals with the VEGF inhibitors did not indicate that hypertension and kidney toxicity would be a concern for the clinic, although some minimal effects on blood pressure were seen with a few of the VEGFR tyrosine kinase inhibitors, and an antidiuretic effect was seen in a renal safety pharmacology

study. Since hypertension is consistently observed in the clinic with all VEGF inhibitors, it is clearly directly related to the mechanisms of action; however, this and other VEGF-related events such as thrombotic and hemorrhagic events and GI perforation may be intensified by concurrent pathological conditions. The small molecule VEGFR tyrosine kinase inhibitors also exhibit some additional toxicities which bevacizumab does not. These toxicities may be due to inhibition of other tyrosine kinases or may be "off-target" toxicities.

Inhibitors of EGFR signaling (erlotinib, gefitinib, lapatinib, vandetanib, cetuximab, panitumumab) primarily exhibit toxicity related to the skin as the major organ toxicity and also exhibit gastrointestinal toxicity. These effects are directly related to the pharmacology of inhibiting EGFR signaling. Interstitial lung disease has also been reported for EGFR inhibitors. This was particularly noted for gefitinib (Iressa) which, 2 years after approval by the FDA, had its label revised to limit the use to cancer patients who were currently benefiting, or previously benefited, from treatment, preventing new patients from being prescribed the drug. The enhanced pulmonary toxicity of gefitinib relative to other EGFR inhibitors may be due to modulation of pathways in addition to EGFR tyrosine kinase (Takada et al. 2011; Takada and Matsuo 2012). Similar pulmonary toxicity was not observed in the animal safety pharmacology or toxicity studies with gefitnib or the other EGFR inhibitors. Although decreases in respiratory rate and tidal volume were observed in monkeys with cetuximab, these effects appear to be part of a more generalized infusion reaction to administration of the mouse:human chimeric protein rather than to direct pulmonary toxicity.

It is not clear whether inhibitors or PDGF receptor signaling are associated with cardiotoxicity. There are currently no approved specific PDGF inhibitors, and therefore, it is not clear which, if any of the toxicities seen with the multi-targeted tyrosine kinases, are associated with blocking PGDFR signaling. PDGFRs are expressed ubiquitously, including on cardiomyocytes and on endothelial cells. Mice that are genetically deficient in PDGFR develop severe heart failure following load-induced stress (Chintalgattu et al. 2010).

Infusion reactions are observed in some patients receiving monoclonal antibody therapies. These reactions, which are immunologically mediated, can affect a number of body systems including the cardiovascular system (chest pain, palpitations, hypo- or hypertension, tachy- or bradycardia, arrhythmias, edema, ischemia, cardiac arrest), the CNS (headache, dizziness, confusion), and the respiratory system (dyspnea, bronchospasm). Rash, nausea, vomiting, and arthralgia are also possible signs of infusion reactions. Infusion reactions occur most frequently with the first dose administration but can usually be managed clinically. Animals are very poor predictors of human immunological reactions, and therefore, neither the safety pharmacology studies nor the general toxicity studies are able to predict the potential for inducing these reactions in patients.

In summary, anticancer agents are associated with a number of toxicities including cardiovascular toxicity. The safety pharmacology studies conducted in normal animals are able to detect the potential to increase QT Interval but have not been able to identify some of the other toxicities that develop with prolonged use or in the

presence of concomitant treatment or comorbidities. Because of the seriousness of cancer, the finding of a positive signal in a safety pharmacology study is unlikely to be the cause for terminating the development of a program. The findings may, however, affect the starting dose, the dose escalation scheme, and the safety monitoring in the clinical trial. The recently issued ICH S9 guidance allows for the safety pharmacology assessments (ECG and detailed clinical observations) to be conducted as part of the non-rodent general toxicity studies. This provision reduces the number of animal studies needed to support clinical dosing. This strategy has generally been the default for large-molecule biopharmaceuticals partly because they are cross reactive only in nonhuman primates and also because they are highly selective in their actions, and off-target toxicities are rare. The experiences with the anticancer agents have indicated that many of the cardiac toxicities may be directly related to the mechanism of action of the therapeutic. Therefore, specifically designed pharmacology studies may provide useful safety information for the clinic in addition to the standard toxicology and safety pharmacology studies.

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Part V

Clinical Safety Pharmacology

Clinical ECG Assessment

Borje Darpo

Contents

Abstract

With the adoption of the ICH E14 guidance, the thorough QT/QTc (TQT) study has become the focus of clinical assessment of an NCE's effects on ECG parameters. The TQT study is used as a guide to the liability of a drug to

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cause proarrhythmias on the basis of delayed cardiac repolarization. Around 300 TQT studies have been performed since 2005 and through interactions between sponsors and regulators, especially FDA's Interdisciplinary Review Team (IRT) for QT studies. These studies can today be performed more effectively and with great confidence in the generated data. This chapter will discuss technical features and the design and analysis of TQT studies, how assay sensitivity is demonstrated, and examples from recently conducted studies. ECG assessment for drugs that cannot be safely given to healthy volunteers is also addressed, and examples from studies in cancer patients and in healthy volunteers with tyrosine kinase inhibitors are discussed. The TQT study is resource intensive and designed to solely evaluate whether an NCE prolongs the QTc interval. If data with similar confidence can be generated from other studies that are routinely performed as part of the clinical development, this would represent a more optimal use of human resources. Methods and approaches to increase the confidence in ECG data derived from "early QT assessment" in single-ascending/multiple-ascending dose studies are therefore discussed, and a path toward replacing the TQT study using these approaches will be outlined.

Keywords

Healthy subjects • Precision • Publications • QT method • QTc • Sample size • Thorough QT studies • Variability

Abbreviations

CI	Confidence interval
E14	ICH Harmonized Tripartite Guideline E14: The Clinical Evaluation of QT/QTc Interval Prolongation and Proarrhythmic Potential for Non-Antiarrhythmic Drugs
ICH	International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
IRT	FDA's Internal Review Team for QT studies
ER	Exposure-response
LB	Lower bound
MAD	Multiple-ascending dose
MTD	Maximum tolerated dose
NCE	New chemical entity
PK	Pharmacokinetic
QTc	Heart rate-corrected QT interval
ΔQTc	Change-from-baseline QTc
ΔΔQTc	Placebo-corrected change-from-baseline QTc
QTcF	QT heart rate-corrected according to Fridericia

QTcI	QT subject-specific heart rate correction
SAD	Single ascending dose
SD	Standard deviation
TM	Time-matched
Tmax	Time of peak plasma level
TQT	Thorough QT/QTc study
UB	Upper bound

1 Introduction

As part of the development program of a new chemical entity (NCE), there is an expectation that the effect of the on ECG parameters should be well characterized. With the adoption of the International Conference on Harmonisation (ICH) E14 guidance in 2005, the center piece of this evaluation has become the thorough QT/QTc (TQT) study, typically performed in healthy volunteers (Darpo 2010; ICH Harmonized Tripartite Guideline E14 2005). Despite the referral to only one of several ECG parameters, the QTc interval, it has become increasingly apparent that this study also can and should assess the effect of the NCE's effect on heart rate and the PR and QRS intervals, since these variables may also be adversely affected. This chapter will discuss features of the TQT study and address definitive ECG assessment for drugs that cannot be safely administered to healthy volunteers, such as many oncology drugs. Lastly, methods and approaches to increase the confidence in ECG data derived from "early QT assessment" in single-ascending/multiple-ascending dose (SAD/MAD) studies will be described, and a path toward replacing the TQT study using these approaches will be outlined.

It should be borne in mind that the objective of ECG assessment in healthy volunteers is limited to the evaluation of the drug's effect on ECG parameters, such as the heart rate-corrected QT interval (QTc). In this context, drug-induced QT prolongation is regarded as a biomarker for proarrhythmic risk in susceptible patients, and testing in healthy volunteers has the purpose of detecting drugs that have a sufficiently large effect to warrant further characterization in the targeted patient population (ICH E14 Questions & Answers 2012). For drugs with a "positive TQT study," i.e., a study in which an effect on QTc exceeding 10 ms cannot be excluded, further ECG monitoring is required in late-stage development. Objectively verified proarrhythmias are however very rarely observed in development programs of normal size or even in programs up to 20,000 patients, with a few notable exceptions with more potent QT-prolonging drugs (Caprelsa US NDA 022405 2011; Darpo 2007). Even in the absence of demonstrated proarrhythmic events, drugs with a more pronounced QTc effect, i.e., above 20 ms, are generally regarded as proarrhythmic by regulators and come with warnings and precautions in the label (Park et al. 2013).

This chapter intends to discuss the assessment of changes of ECG parameters as part of the drug development, with focus on studies and approaches specifically designed to evaluate drug-induced changes. Recent approvals of drugs with a relatively potent QT-prolonging effect, e.g., vandetanib (Thornton et al. 2012) and, most recently bedaquiline, illustrate that QT prolongation and the proarrhythmic risk associated therewith are a part of the benefit/risk assessment that all drugs must undergo; an informed discussion on this topic requires an in-depth understanding of the targeted indication and of the drug's effectiveness and is therefore out of scope for this text.

2 The Thorough QT/QTc (TQT) Study

2.1 The TQT Study

The "International Conference on Harmonisation (ICH) of Technical Requirements for Registration of Pharmaceuticals for Human Use" issued the E14 clinical guidance in May 2005: "The Clinical Evaluation of QT/QTc Interval Prolongation and Proarrhythmic Potential for Non-Antiarrhythmic Drugs" (ICH Harmonized Tripartite Guideline E14 2005). The guidance was implemented in Europe and in the United States during the same year, but only more recently in Japan (November 2010). The TQT study is a dedicated study with the primary objective to quantify the effect of the NCE on the QT interval. In 2007, the US FDA formed an Interdisciplinary Review Team for QT studies (IRT) with the responsibility to oversee the clinical assessment of QT prolongation for all drugs that the agency reviews. The IRT serves in an advisory function to the reviewing divisions, reviews all TQT study protocols, and advises sponsors on the design of the study and supports the reviewing division in the interpretation of data. Individual members of the IRT have shared their experience at various meetings (e.g., DIA meetings) and through publications (see, e.g., Florian et al. 2011; Garnett 2012; Garnett et al. 2008; Malik et al. 2010; Zhu et al. 2010); this level of transparency has been of great value for sponsors and most likely also for regulators in other regions. Based on these interactions and experience from a large number of completed TQT studies (more than 300, July 2013), a standard for TQT studies has evolved. In line with this accumulated experience, clarifications to the E14 guidance have been issued in June 2008 and April 2012 (ICH E14 Questions & Answers 2012). Even so, there is clearly more than one way of performing a TQT study that will be accepted, and there are also areas which remain debated at the present time, e.g., how to best correct the QT interval for heart rate changes (Garnett et al. 2012).

To serve as a basis for discussion on the design of recent TQT studies, a PubMed search was done (December 31) using the search terms "thorough QT study 2012" and "thorough QTc QT study 2012." Eighteen TQT studies published in 2012 were identified; the key features of these studies and the response to the positive control, in all cases moxifloxacin, are summarized in Table 1. The limitations of this approach should be acknowledged: The list is most likely not complete; only a

Table 1 Thorough QT studies published in 2012

	Drug	References	Indication	Population (*n*)	Design	Treatments	Main result	Peak moxi ΔΔQTc[a] ms	SD of ΔQTc # of ECG replicates/ timepoint
1	Betrixaban	Morganroth et al. (2013)	Antithrombotic	HV; M + F $n = 96$	4-way XO; SD	T, ST, M, P	Negative	11.5 ± 2.7	SD = 11.3 ms with $n = 96$ for M/P #ECG: 3
2	Vismodegib	Graham et al. (2013)	Basal cell carcinoma	HV; F $n = 60$ (20 in 3 groups)	Parallel; nested XO MD; 7 days	T, M, P	Negative (UB of CI 10.0 ms)	19.0 ± 3.9	SD = 10.5 ms with $n = 40$ for M/P #ECG: 3
3	Bitopertin	Hofmann et al. (2012)	Schizophrenia	HV; M $n = 169$ (56–57/3 groups)	Parallel; nested XO MD; 10 days	T, ST, M, P	Negative	10.6 ± 3.7	SD = 11.9 ms with $n = 57$ for M/P #ECG: 3
4	Inhaled dihydroergotamine	Kori et al. (2012)	Migraine	HV, M + F $n = 54$	3-way XO SD	ST, M, P	Negative	11.3 ± 2.35	SD = 7.4 ms with $n = 54$ for M/P #ECG: 3
5	Exenatide	Darpo et al. (2013)	Type 2 diabetes mellitus	HV; M + F $n = 74$	3-way XO MD; 3 days	T + ST, M, P	Negative	10.9 ± 2.2	SD = 8.1 ms with $n = 74$ for M/P #ECG: 4
6	Sugammadex	de Kam et al. (2012)	Reversal of neuromuscular blockade	HV, M + F $n = 58$	4-way XO SD	T, ST, M, P	Negative	18.6 ± 3.6[b] 400 mg IV	SD = 11.7 ms with $n = 58$ for M/P #ECG: 3
7	Semagacestat	Zhang et al. (2012a)	Alzheimer disease	HV; M + F $n = 54$	4-way XO SD	T, ST, M, P	Negative	12.9 ± 1.9	SD = 5.8 ms with $n = 52$ for M/P #ECG: 4

(continued)

Table 1 (continued)

	Drug	References	Indication	Population (*n*)	Design	Treatments	Main result	Peak moxi $\Delta\Delta QTc^a$ ms	SD of ΔQTc # of ECG replicates/ timepoint
8	Bilastine	Tyl et al. (2012)	Allergic rhino-conjunctivitis	HV; M + F $n = 30$	5-way XO MD; 4 days	T, ST, T + keto, M, P	Negative	19.9 ± 2.9 MD 3 days	SD = 6.7 ms with $n = 30$ for M/P #ECG: 3
9	Lersivirine	Vourvahis et al. (2012)	HIV infection	HV; M $n = 48$	3-way XO SD	ST, M, P	Negative	15.3 ± 1.85	SD = 5.5 ms with $n = 48$ for M/P #ECG: 3
10	Gabapentin enacarbil	Chen et al. (2012)	Restless legs syndrome	HV; M + F $n = 50$	4-way XO SD	T, ST, M, P	Negative	7.5 ± 2.2	SD = 6.6 ms with $n = 50$ for M/P #ECG: 3
11	Midostaurin	del Corral et al. (2012)	TKI; acute myeloid leukemia	HV; M + F $n = 192$ 44, 68, 80/3 groups	Parallel MD; 3 days	T, M, P	Negative	10.7 ± 4.3	SD = 13.1 ms with $n = 43$/ 64 for M/P #ECG: 3
12	JNJ-Q2	Eichenbaum et al. (2012)	Antibiotic	HV; M + F $n = 58$	4-way XO MD; 4 days	T, ST, M, P	Positive	11.5 ms (CI not given)	NA
13	Dapagliflozin	Carlson et al. (2011)	Type 2 diabetes mellitus	HV, M $n = 50$	4-way XO SD	T, ST, M, P	Negative	11.5 ± 2.3	SD = 6.9 ms with $n = 50$ for M/P #ECG: ~10

14	Mirabegron	Malik et al. (2012)	Overactive bladder	HV, M + F $n = 352$ 88/4 groups	Parallel group with XO vs. placebo MD; 10 days	T, ST, M, P	Positive for females	Males, 9.14 ± 1.56 Females, 8.58 ± 2.21	SD = 5.0[c] ms with $n = 42$ for males and $n = 37$ for females for M/P XO #ECG: 5
15	Insulin; food methodology study	Taubel et al. (2013)	Diabetes mellitus	HV; M + F $n = 32$	2-way XO SD	Insulin, food, M, P	Negative	14.4 ± 2.5	SD = 6.0 ms with $n = 32$ for M/P #ECG: 3
16	Bupivacaine	Naseem et al. (2012)	Local anesthetic	HV, M + F $n = 49$	5-way XO SD	T, ST, M, P	Negative	11.3 ± 2.5	SD = 7.4 ms with $n = 48/49$ for M/P #ECG: 3
17	Saquinavir	Zhang et al. (2012b)	HIV infection	HV, M + F $n = 66$	4-way XO MD; 3 days	T, ST, M, P	Positive	12.2 ± 4.1	SD = 13.5 ms with $n = 60$ for M/P #ECG: 3
18	Prucalopride	Mendzelevski et al. (2012)	Chronic obstipation	HV, M + F $n = 120$ 60/ 2 groups	Parallel; nested XO MD 15 days	T, ST, M, P	Negative	13.38 ± 4.24[d]	SD = 14.0 ms with $n = 60$ for M/P #ECG: 3

The SD of ΔQTc was estimated based on the assumptions that the 90 % CI for peak moxifloxacin ΔΔQTc was calculated from a 2-sample *t*-test with equal variance for moxifloxacin and placebo data

HV, healthy volunteers; M, males; F, females; XO, crossover; SD, single dose; MD, multiple dose; T, therapeutic dose; ST, supratherapeutic dose; P, placebo; M, moxifloxacin, single oral dose of 400 mg; nested XO, nested crossover comparison for moxifloxacin/placebo within a parallel group study; T + keto, therapeutic dose concomitant with ketoconazole. TKI, tyrosine kinase inhibitor; CI, confidence interval; LB, lower bound. Negative: upper bound of CI < 10 ms for doses studied

[a]QTcI or QTcF; confidence interval (CI) calculated from given lower or upper bound

[b]Intravenous moxifloxacin 400 mg; CI not detailed; visually estimated from graph

[c]Average across male and female group

[d]Personal communication from the authors

small fraction of TQT studies are published and most likely subject to selection bias, and some studies may have been conducted several years ago. Despite this, the sample seems representative of the types of TQT studies that have been conducted during the last few years.

2.2 Timing of the TQT Study

There is no formal regulatory requirement on when the TQT study should be performed, but in most cases, the results are expected to be available before initiating late-stage, confirmatory efficacy trials; the consequence of not having these results available before starting phase III trials is that the level of ECG monitoring will be as intense as if the NCE were found to be positive in the TQT study. On the other hand, it is critical to have sufficient knowledge of the pharmacokinetic (PK) characteristics of the NCE in the targeted patient population, in particular data from patients with impaired clearance due to intrinsic or extrinsic factors, such as drug interactions. The timing of the TQT study will also be influenced by "QT signals" determined from safety pharmacology studies; for a drug with an unambiguous nonclinical QT signal, it may be important to determine the level of QTc effects in humans early in clinical development, as this will have a bearing on the benefit/risk assessment and thereby the potential viability of the project. For NCEs targeting more severe medical indications, e.g., oncology drugs, in particular when no other effective therapy exists, it may be preferable to conduct the TQT at a stage when some confidence in the clinical effectiveness has been gained. There have been advancements in the ability of chemists to design NCEs without hERG inhibition (Leeson and Springthorpe 2007), but even so, NCEs from certain pharmacological classes, e.g., fluoroquinolone antibiotics, often demonstrate QT liability. For such classes, it may also be advisable to perform the TQT relatively early in the program.

2.3 Design Considerations

When considering the clinical study trial design, it is critical to know the variability that exists between study types. The "within-subject" variability is lower than the "between-subject" variability, and a crossover-designed TQT study is therefore more efficient than a parallel-designed study and as such requires a slightly reduced sample size and, obviously, fewer subjects. This was pointed out in the E14 guidance and has been emphasized by the IRT on several occasions (Zhang 2012). When the NCE needs to be dosed for more than approximately a week to reach sufficiently high steady-state plasma levels due to accumulation, or needs to be titrated based on tolerability, a parallel-designed study is preferable. The study duration, i.e., from first-subject-in to last-subject-out, can also be shorter with a parallel-designed study, provided the clinical site can handle sufficiently large cohorts concurrently. A parallel-designed study can be made more effective as

compared to the standard design with four treatment groups (i.e., placebo, moxifloxacin, and a therapeutic and supratherapeutic dose of the NCE), by using a nested crossover comparison within a combined placebo/moxifloxacin group, as suggested by Dr. Joanne Zhang from the IRT (Zhang 2009). In this nested design, half of the subjects in the combined placebo/moxifloxacin group are dosed with the positive control on the first day of treatment, and in the other half of subjects, dosing occurs on the day after the last treatment day. At the time of data analysis, results from both halves are combined for the placebo-component of the ΔΔQTc effect of the NCE, which therefore is not impacted by this design (for details on the analysis, see Darpo 2010). The advantage of this design is that the number of subjects in the placebo and moxifloxacin groups is reduced by 50 %, even though the number of days on which ECGs are analyzed remains essentially the same. This design now seems to be generally accepted and was used in 3 of 5 parallel-designed TQT studies published in 2012 (Graham et al. 2013; Hofmann et al. 2012; Mendzelevski et al. 2012); the remaining 13 were crossover studies (Table 1).

Study Population The concept that underlies the TQT study is that if an NCE causes QT prolongation and proarrhythmias in patients, then the QT effect can be demonstrated in healthy volunteers, if sufficiently high doses (i.e., multiples above the therapeutic dose) of the NCE are given. The challenges associated with TQT studies performed in patients are numerous and are the result of many factors including the number of clinical trial sites, adequate training of investigators, and higher incidence of cardiovascular disease leading to larger variability of ECG interval measurements. Accordingly, the vast majority of TQT studies today are performed in healthy volunteers provided the NCE can be safely dosed to this population. There are however occasional examples of "TQT-like" studies conducted in a relatively limited patient population, such as Parkinson's disease, where group sizes are small ($n = 130$) with stringently controlled experimental conditions (Malik et al. 2008).

Initially, there were no requirements on gender or ethnicity considered in the conduct of the TQT study. Since the adoption of the E14 guidance, gender has been discussed twice in the Q&A documents released in June 2008 and April 2012 (ICH E14 Questions & Answers 2012). It is known that women have a somewhat longer QTc interval than men (Burke et al. 1997; Rautaharju and Zhang 2002; Sarapa et al. 2004), and it has been shown that the degree of drug-induced QTc prolongation may vary in different phases of the menstrual cycle (Rodriguez et al. 2001). It is also well documented that women are at higher risk for the development of proarrhythmias caused by drugs with an effect on cardiac repolarization, i.e., drugs that prolong the QTc interval (Bednar et al. 2002; Ebert et al. 2000; Makkar et al. 1993). It would therefore seem reasonable to assume that women also react with a larger degree of QTc prolongation than men at the same plasma exposure of a drug, and there are a few documented examples thereof (Rodriguez et al. 2001; Benton et al. 2000; Shin et al. 2007; Darpo et al. 2012). For drugs with only a mild effect on the QT interval, it has been difficult to demonstrate a gender difference in sensitivity for the drug-induced QTc prolongation. In a

pooled analysis of data from 2 studies in healthy Japanese and Caucasian volunteers who were dosed with levofloxacin, age and gender did not have an effect on the level of QT prolongation when analyzed with a linear exposure-response (ER) model (Sugiyama et al. 2012). Sex differences in QTc prolongation for moxifloxacin were investigated in a pooled analysis of 20 TQT studies that used moxifloxacin as a positive control (Florian et al. 2011). Women had approximately 40 % higher moxifloxacin peak plasma levels than men and a statistically significant larger peak QTcF effect with a placebo-corrected ΔQTcF of 12.4 ms (confidence interval (CI): 11.1–13.7 ms) compared to 9.1 ms (CI: 8.1–10.1 ms) in men. There was however no difference in the slope estimate for the exposure-response (ER) relationship, which means that the observed difference in QTcF prolongation can be explained by the differences in plasma levels. In line with these considerations, the latest version of the Q&A document states Question 8 in (ICH E14 Questions & Answers 2012):

The thorough QT study is primarily intended to act as a clinical pharmacology study in a healthy population using a conservative primary objective defining the drug's effect on QT. It is unlikely that any of a variety of baseline demographic parameters would introduce a large difference in QT response to a drug in subpopulations defined by factors such as age, co-morbidity, and gender that is not explained by exposure. It is encouraged, but not mandatory, to include both men and women in the thorough QT study. Analyses of concentration response relationship by sex can be helpful for studying the effect of the drug on QT/QTc interval in cases where there is evidence or mechanistic theory for a gender difference. However, the primary analysis of a thorough QT study should be powered and conducted on the pooled population. If the primary analysis is negative and if there is no other evidence suggesting gender differences, subgroup analysis by sex is not expected.

Among the 18 TQT studies published in 2012, the majority ($n = 14$) were conducted in both male and female healthy volunteers, whereas three studies were conducted only in males (Carlson et al. 2011; Hofmann et al. 2012; Vourvahis et al. 2012) and only one in females (Graham et al. 2013).

Dose A high, supratherapeutic dose of the NCE, which results in plasma levels in excess of what would be observed in patients with impaired clearance of the drug, should be used in the TQT study. The E14 states: "If not precluded by considerations of safety or tolerability due to adverse effects, the drug should be tested at substantial multiples of the anticipated maximum therapeutic exposure." The overriding principle is that plasma levels achieved with the supratherapeutic dose should exceed the "worst-case scenario" in patients, taking into account both intrinsic (e.g., renal impairment) and extrinsic factors (e.g., drug interactions). As an example, for NCEs that are CYP 3A4 or 2D6 substrates, the achieved exposure must exceed that observed with concomitant administration with potent 3A4 inhibitors, and in 2D6 poor metabolizers (Abbas et al. 2012; Boyce et al. 2012; Chaikin et al. 2005; Dalen et al. 2010; Malhotra et al. 2007; Robert et al. 2007; Tyl et al. 2012; Zhu et al. 2010). For a renally cleared drug, plasma levels that are only

observed in patients with severe renal impairment (i.e., where the glomerular filtration below 30 mL/min) may not have to be covered in the TQT study if the drug is contraindicated in this population. The selection of the supratherapeutic dose is the most common reason for discussions between regulators and sponsors, and failure to study a sufficiently high dose has occasionally led to a requirement for a repeat TQT study; this is therefore an important part of the dialogue between sponsors and regulators before the TQT study is initiated. It should also be borne in mind that formulation changes that result in substantially higher plasma levels of a drug may require conduct of an additional TQT study. A recent example is exenatide, a GLP-1 agonist intended for the treatment of type 2 diabetes mellitus. Two separate TQT studies had to be conducted for two different exenatide formulations (Byetta® and Bydureon®), both of which were negative (Darpo et al. 2013; Linnebjerg et al. 2011). The first TQT study was conducted with the daily subcutaneous formulation (Byetta®) at the approved therapeutic dose (10 μg twice daily). On chronic administration with a therapeutic dose of the later developed once-weekly formulation (Bydureon®, 2 mg once weekly), exenatide plasma levels were at least twofold higher than those with the daily formulation and even higher in patients with impaired renal function. Accordingly, the FDA required to conduct a second TQT study, in which substantially higher plasma levels were achieved through an IV infusion of the drug (Darpo et al. 2013).

In most TQT studies to date, both a therapeutic and a supratherapeutic dose of the NCE have been studied. There are, however, a few studies in which only a supratherapeutic dose of the NCE was included (Iwamoto et al. 2008; Krishnaswami et al. 2011; Vourvahis et al. 2012; Zhang et al. 2007). This approach is obviously sufficient if results are clearly negative. Most sponsors tend however to also include the therapeutic dose, in case the high dose is "slightly positive." It can be argued that the effect of the therapeutic dose can be projected by ER analysis, and it would therefore be sufficient to include only a supratherapeutic dose, but this has so far not gained widespread acceptance. There is no requirement per se on dosing to steady state of the NCE, and single doses can be used in the TQT study, provided that a sufficiently high exposure of both parent and major metabolites can be achieved. If there are slowly appearing metabolites, which require many days of dosing to achieve sufficiently high exposure, multiple dosing is warranted.

2.4 Analysis of the TQT Study Results

The objective of the TQT study is to demonstrate that an NCE does not prolong the QTc interval by more than 5 ms, as evidenced by the upper bound (UB) of the 2-sided 90 % CI of the placebo-adjusted change-from-baseline QTc (ΔΔQTc) being below 10 ms.

In crossover-designed TQT studies, the baseline assessment can be made either through time-matched recordings on a full baseline day before each treatment period (time-matched baseline) or through a limited number of recordings (e.g.,

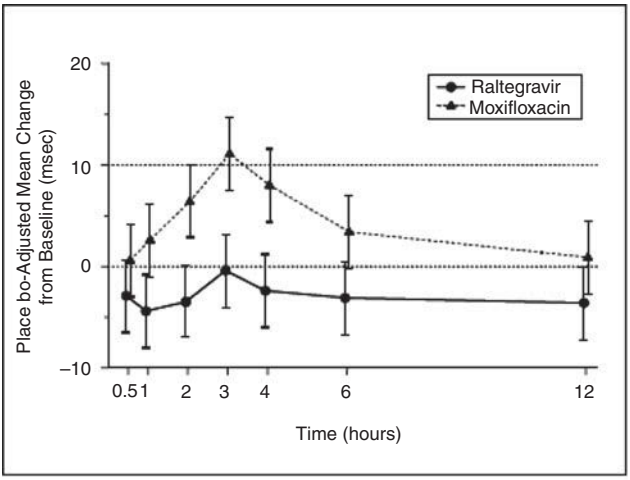
3 timepoints) before each treatment period (predose baseline). Based on the experience from several sponsors (Bloomfield et al. 2008), core ECG laboratories, and the IRT (Zhang and Machado 2008), it is fully sufficient to use a predose baseline since adjustments for subject- and study-specific diurnal variation are accounted for by the inclusion of a separate placebo treatment period (see question 6 in E14 Q&A document (ICH E14 Questions & Answers 2012). For parallel-designed TQT studies, a full baseline day is still the most widely used approach and change-from-baseline QTc (ΔQTc) is then calculated by comparing the QTc value for each timepoint at baseline and post-dosing ("time-matched"). There are some data suggesting that results would be the same and the variability lower if baseline was generated through averaging of all values from a full baseline day (Sun et al. 2012), then more research and analyses across TQT studies can be expected (Lu 2013).

The criterion for a negative TQT study specifically is that the UB of the 90 % CI of the ΔΔQTc estimate is below 10 ms, which applies to *all* post-dosing timepoints. Since the analysis uses a non-inferiority approach, there is no need for adjustment for multiplicity in this part of the analysis (Stockbridge et al. 2012; Tsong et al. 2008, 2010; Zhang and Machado 2008). Figure 1 shows two examples of clearly negative TQT studies (Tyl et al. 2012; Iwamoto et al. 2008).

The role of the positive control is to demonstrate the study's ability to detect a small effect on the QT interval, establishing assay sensitivity. In an overwhelming majority of TQT studies, moxifloxacin, a fluoroquinolone antibiotic with a mild QT-prolonging effect (Culley et al. 2001), has been used. In the studies published in 2012, all used moxifloxacin as the positive control; 16 of 18 studies used a single oral dose of 400 mg, 1 used multiple dosing for a 3-day duration (Tyl et al. 2012), and one used an IV infusion (de Kam et al. 2012). In most studies, moxifloxacin has caused a larger peak effect than 5 ms, more in the range of 8–15 ms (Garnett et al. 2008). The differences in peak response across studies is however quite striking and ranges between 7.5 ± 2.2 ms (Chen et al. 2012) and 19.0 ± 3.9 ms (Graham et al. 2013) in the studies published in 2012 that used a single oral dose of 400 mg moxifloxacin. The criteria for demonstrating assay sensitivity with moxifloxacin have been addressed in the first round of the E14 Q&A document [Question 1 in ICH E14 Questions & Answers (2012)] and subsequently clarified through interactions between IRT and sponsors. The criteria include:

(a) The lower bound (LB) of the 90 % CI of ΔΔQTc should be above 5 ms for at least one prespecified post-dose timepoint.
(b) The peak ΔΔQTc should be within the range of responses seen in similar studies, i.e., about 8–16 ms, even though the exact cutoff points are less clear.
(c) The mean peak ΔΔQTc should be observed between 1 and 4 h post-dose and thereafter declines. Note that lately, the IRT has also asked for a timepoint earlier than the peak effect.

Panel A



Panel B

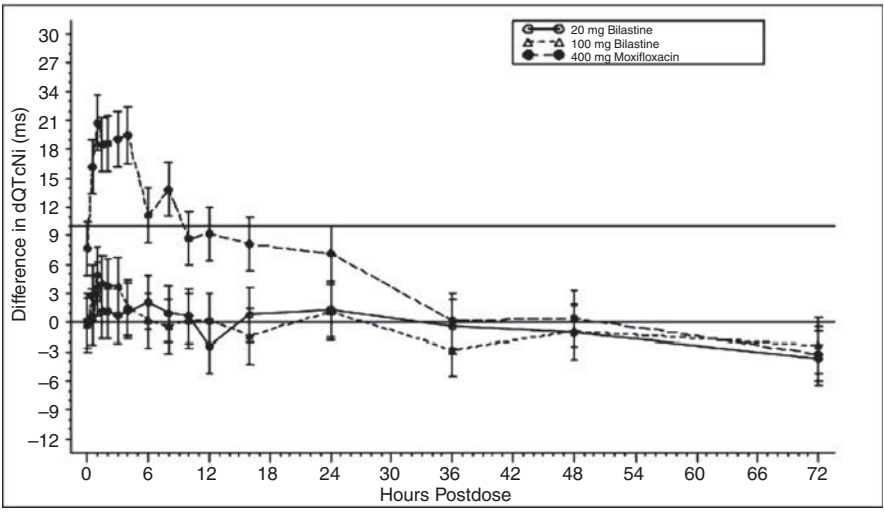


Fig. 1 Panel (**a**). An example of a negative TQT study where ΔΔQTcF (mean ± 90 % CI) is determined after dosing with a single-dose of 1,600 mg raltegravir (Isentress) and 400 mg moxifloxacin. For raltegravir, the UB of the CI is below 10 ms at all post-dosing timepoints, thus demonstrating a negative TQT study. After dosing with moxifloxacin, the LB of the CI is above 5 ms at the observed peak effect at 3 h, thereby demonstrating assay sensitivity for the study. *Reproduced from Iwamoto et al. (2008) with permission from J Clin Pharmacology, American College of Clinical Pharmacology and John Wiley & Sons, Inc.* Panel (**b**). An example of a negative TQT study where ΔΔQTcNi (individualized QTc; mean ± 90 % CI) is determined after 4 days of dosing with bilastine (Bilaxten) 100 mg and 200 mg once daily and 400 mg moxifloxacin for 3 days. Bilastine produces a clearly negative effect, whereas the moxifloxacin response is relatively high (19.9 ± 2.9 ms), and the LB of the CI is above 5 ms at multiple post-dosing timepoints. *Reproduced from Tyl et al. (2012) with permission from J Clin Pharmacology, American College of Clinical Pharmacology and John Wiley & Sons, Inc.*

Since this analysis is about detecting an effect, an adjustment for multiplicity has to be made, and it is therefore advisable to limit the number of timepoints to those around the peak plasma concentration (Tmax) of moxifloxacin, usually 1–3 h post-dose. A number of methods have been used for the multiplicity adjustment and accepted by the IRT, of which the Hochberg procedure seems to be the least conservative (Hochberg and Benjamini 1990). Even though the primary model-based analysis of ΔΔQTcF on moxifloxacin can be restricted to a few timepoints to avoid diluting the statistical power, there is an expectation that more timepoints are analyzed descriptively to ensure that the moxifloxacin response diminishes over time. The studies shown in Fig. 1 are good examples of studies in which the criteria for demonstration of assay sensitivity were clearly met. In Fig. 2, panels (a) and (b) describe two examples of relatively recently published studies in which the criteria for moxifloxacin assay sensitivity that were not met are shown (March and Cardi 2009; Morganroth et al. 2010). The peak effect of ΔΔQTc after moxifloxacin (a single oral dose of 400 mg) was comparable with other studies, but the precision of the ΔΔQTc estimate is poor, which resulted in very wide limits of the 90 % CI with the LB below 5 ms at all timepoints. Based on the IRT's experience, the assay sensitivity test with moxifloxacin has failed in about 5 % of cases (Garnett 2012), but for the majority of the studies, the confidence in the data is very high.

Initially, the IRT mandated blinding of the positive control, but this requirement was later dropped based on an internal IRT review of TQT studies presented in 2008 (Garnett et al. 2008) and subsequent discussions in the E14 Implementation Working group [Question 7 in ICH E14 Questions & Answers (2012)]. Exceptions exist: when the nested crossover moxifloxacin/placebo comparison is used in parallel-designed TQT studies, moxifloxacin has to be blinded to protect the blinding of placebo.

2.4.1 Sample Size

The difference in sample size is quite striking among studies published in 2012 (see details in Table 1), ranging from 60 (Graham et al. 2013)[1] to 352 subjects in parallel-designed studies (Malik et al. 2012) and from 32 subjects for 2 treatment periods [(Taubel et al. 2013); methodology study] to 96 subjects for 4 periods (Morganroth et al. 2013) for crossover studies.

When calculating the sample size for a TQT study, the power of the study to exclude a QTc effect above 10 ms, the underlying assumed effect of the NCE (often 3–5 ms), and the variability of QTc are important factors that need to be factored into the equation. Using the same assumptions (90 % power and 3 ms assumed effect), the required sample size varies from approximately 50 subjects with a SD of ΔQTc of 8 ms to around 100 subjects with a SD of 12 ms, which underlines the importance of tightly controlled experimental conditions and ECG methodologies (Darpo et al. 2011). The variability of the moxifloxacin peak ΔQTc can be estimated using the width of the 90 % CI, based on the assumptions that it was

[1] This study was powered to exclude a 20 ms QTc effect.

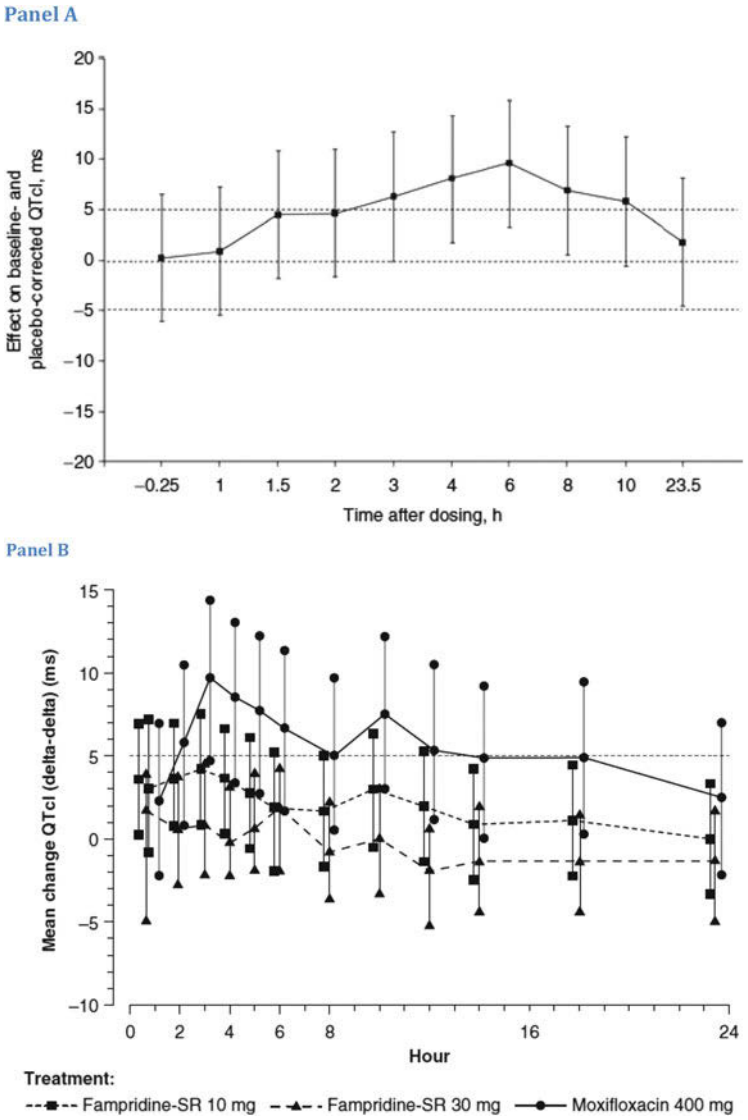


Fig. 2 Panel (**a**). The moxifloxacin response (single 400 mg oral dose, ΔΔQTcI; mean ± 90 % CI; $n = 47$) in a TQT study with an alfa-receptor blocker, silodosin (Rapaflo). The peak magnitude of the moxifloxacin response is similar as compared to other TQT studies, but the time-to-maximum ΔΔQTcI occurs somewhat later. Due to poor precision of the ΔΔQTc estimate with unusually wide CIs, none of the LB exceeds 5 ms. The assays sensitivity test, as defined by ICH E14, has thereby failed. *Reproduced from Morganroth et al. (2010) with permission from Clinical Pharm & Therapeutics, American Society for Clinical Pharmacology and Therapeutics and Nature Publishing Group*. Panel (**b**). The moxifloxacin response (single 400 mg oral dose ΔΔQTcI, mean ± 90 % CI; $n = 51$) in this example of a TQT study conducted for fampridine (Ampyra) peaks at 9.4 ms with very wide CIs, for which the LB does not exceed 5 ms at any timepoint

calculated from a 2-sample *t*-test with equal variance of the moxifloxacin and placebo data. These assumptions are not always entirely true, but the approach gives a fair estimate of the QTc variability using a consistent methodology across studies. Since the estimated variability is calculated from the moxifloxacin ΔΔQTc, it is independent of the NCE and can be used for comparisons between studies irrespective of the size. It should be emphasized that QTc variability results from many different factors. Some of the more important non-NCE-related factors include the experimental conditions at the clinical trial site, ECG recording/extraction methods, and ECG interval measurement techniques. The largely observed difference in QTc variability across the studies published in 2012 is surprising and cannot easily be attributed to one single factor: the standard deviation of ΔQTc ranged from 5.5 ms to 14.0 ms (Table 1). Expectedly, many of the studies with the lowest variability are single-dose crossover studies (7 of 8 with standard deviation below 8 ms), and the ones with the highest variability are all parallel group designed. With the exception of the TQT study with dapagliflozin, which utilized a technique proprietary to the sponsor, most studies used manual or semiautomated measurement techniques. Given the differences in variability with similar ECG methodologies, these data also suggest an important role for the clinical conduct and the level of experimental control at the clinical trial site, in addition to the study design (crossover or parallel).

2.5 Correction of the QT Interval for Heart Rate Changes

There are numerous ways of correcting changes in the heart rate to obtain the corrected QT interval (QTc), and there is no clear consensus on the preferred algorithm (Malik 2001). The limitations of Bazett's QT correction (QTcB) are widely acknowledged, since this algorithm overcorrects the QT interval with increasing heart rate, thereby producing a false-positive QTc prolongation. Consequently, it is no longer a requirement to report this interval for TQT studies [Question 11 in ICH E14 Questions & Answers (2012)]. For drugs without clear effect on the heart rate, it has been the experience of the IRT, and of many sponsors, that QTcF works well (Zhang 2012). For these drugs, there does not seem to be much of an advantage to use a subject-specific QTcI derived from supinely resting drug-free data only, which is the standard way of generating QTcI and often results in a correction factor near 0.33 (i.e., very similar to Fridericia, QTcF). Furthermore, the derivation of QTcI is sometimes used to justify an additional full baseline day in crossover-designed studies, which is difficult to defend when there is no added

Fig. 2 (continued) post-dose. Therefore, this study does not establish assay sensitivity. *Reproduced from March and Cardi, Assessment of the cardiac safety of fampridine-SR sustained-release tablets in a thorough QT/QTc evaluation at therapeutic and supratherapeutic doses in healthy individuals. Expert Opin Investig Drugs 18: 1807–1815, copyright 2009, Informa Healthcare. Reproduced with permission from Informa Healthcare*

value of using this correction method. Drugs with an inherent, substantial (e.g., more than 8 bpm peak effect) heart rate effect pose much more of a challenge, and there is no firm guidance. The Cardiac Safety Research Consortium (http://www.cardiac-safety.org/) recently issued a white paper on this topic, which discussed five alternative ways for QT assessment of drugs with a heart rate effect. Methods include "Holter-bin" (Badilini and Maison-Blanche 2005; Malik 2005; Extramiana et al. 2005), QTcI derived from a broad range of QT/RR pairs through continuous Holter recordings at baseline, beat-to-beat analysis (Fossa et al. 2007, 2011), PK/PD modeling with heart rate as a covariate (Li 2008), and assessment of the QT interval at a fixed heart rate through, e.g., submaximal exercise (Demolis et al. 1996, 2000, 2003). The advantages and disadvantages of the methods are discussed, but there is a lack of comparative data across methods. A shared feature of all methods is that baseline QT/RR pairs must be collected from a sufficiently broad range of heart rates, which covers the ranges seen post-dosing with the NCE [see also Question 11 in ICH E14 Questions & Answers (2012)]. When more than one method for heart rate correction is used, it is also advisable to prospectively define the methodology by which the primary endpoint will be chosen.

It was recently suggested by Dr. Joanne Zhang, lead statistician on the IRT, that the variability around the correction factor for the slope estimate should also be taken into account when analyzing ΔΔQTc (Zhang 2012). Obviously, there would be a penalty in terms of wider CIs if the slope is derived from a limited data set according to standard practice and no penalty at all if the choice was to use QTcF, which uses a fixed correction factor of 0.33 ($QTc = QT * RR^{-0.33}$). Since QTcF is regarded as not fully reliable for drugs with a heart rate effect (Garnett et al. 2012), the bottom line is however to use much richer data sets with a broad range of heart rates for the calculation of an "optimized" QTcI.

2.6 ECG Recordings and QT Interval Measurements

The experimental conditions of the TQT study must be stringently controlled and study procedures identical between treatment arms and groups. Several components of the study conduct, which are routinely implemented in TQT studies, have an impact on the variability of the data (Darpo 2010), in addition to the variability of the interval measurements as such (Darpo et al. 2011). Experimental conditions must be strictly standardized with regard to meal intake and composition and physical activity. To minimize heart rate fluctuations, subjects should be supinely resting for at least 10 min in an undisturbed environment at the prespecified timepoints for ECG recordings. The use of continuous 12-lead ECG recordings (Holter's) is preferred as it allows extraction of replicate ECGs around prespecified timepoints with optimal signal-to-noise ratio. Blood draws should always be done immediately after the ECG recording to avoid confounding stress and should be performed in all treatment periods, even though the samples from the placebo and positive control may not be analyzed. A rationale for storing samples from the positive control arm can be that ER analysis sometimes can help explain ambiguous

results. For example, there have been cases with lower-than-expected moxifloxacin QTc effects due to low plasma levels when encapsulation has been used for blinding. Awareness of treatment may introduce a confounding effect on the QT interval, and double-blind administration of placebo/NCE is therefore an absolute requirement that remains in effect. The pharmacokinetic properties of the NCE must be well characterized before the TQT study is initiated (or alternatively, doses up to maximum tolerated (MTD) can be used), and ECG acquisition and blood samples should encompass the anticipated Tmax of the drug and major metabolites (and of moxifloxacin) and at least one timepoint before and several timepoints after Tmax. Often, this can be achieved with 6 to 8 timepoints, and the balance between the number of timepoints and the likelihood of false-positive results needs to be taken into account; this likelihood increases with the number of timepoints. Even so, it is important to also include some late timepoints, e.g., 24 h after dosing, to capture delayed effects including hERG trafficking (Dennis et al. 2012; Ficker et al. 2004; Kuryshev et al. 2005; Ponte et al. 2010). To avoid alterations of autonomic tone, which also has an impact the QTc interval, it is important to avoid timepoints at which subjects may be sleeping, i.e., nighttime.

Averaging replicates of ECG recordings from each timepoint is now standard as it reduces the variability of the QT measurement and therefore increases the power to exclude small QTc effects. With semiautomated methods of ECG measurement, where the computer-based measurements are "overread," i.e., adjusted manually, there are several data sets that demonstrate that the reduction of variability is pronounced when averaging up to triplicates and then levels off [an example is given in Patterson et al. (2005)], and most ECG laboratories today use triplicate ECG recordings at each timepoint (14 studies in Table 1 used 3 replicates (triplicates), 2 studies used 4 replicates, 1 used 5 replicates, and 1 used 10 replicates, respectively). Even though there appears to be a limitation to the extent that variability can be reduced with replicates of intervals measured with a semiautomated technique, this does not seem to hold true for other techniques, which do not blend computer-based and manually performed interval measurements. The utility of fully or partially automated measurement techniques has been compared with manual techniques in a number of studies, and these techniques have been shown to produce similar results when tested on drugs with a QT-prolonging effect (Azie et al. 2004; Darpo et al. 2006; Sarapa et al. 2004, 2009; Fosser et al. 2009). It has also been shown that different techniques generate different absolute QT intervals (Kligfield et al. 2006, 2007) and that some automated techniques consistently demonstrate the same QTc effect measured as change from baseline as manual techniques. The absolute QT interval is of interest in clinical assessment of, e.g., QT prolongation but less important when change from one timepoint to another is the main objective, as in TQT studies.

The variability of the QT measurement (SD of ΔQTc) can be reduced to around 5–6 ms by the use of more computer-intensive techniques with optimization of the ECG extraction and measurement of substantially more beats per timepoint (Dalen et al. 2010; Darpo et al. 2011); see Fig. 3 for an example. There may be concerns regarding the ability of fully automated methods to identify individual patients with

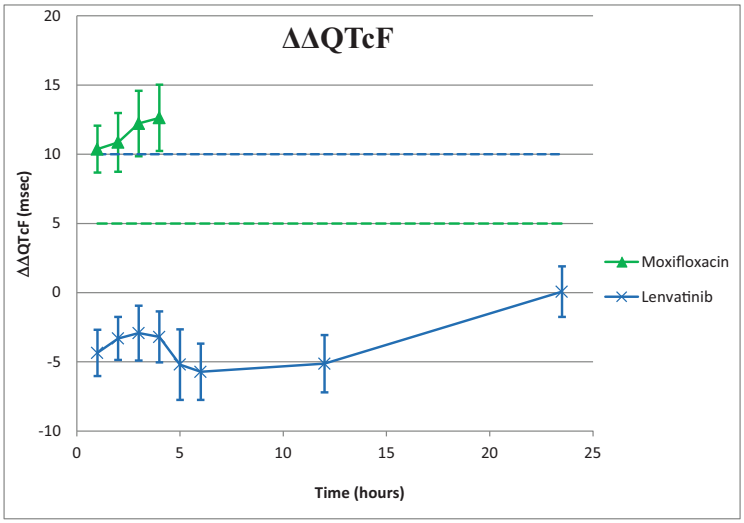


Fig. 3 Placebo-corrected, change-from-baseline QTcF (ΔΔQTcF, mean ± 90 % CI; $n = 52$) after a single 32 mg oral dose of the tyrosine kinase inhibitor, lenvatinib, currently in development for the treatment of various types of cancer and 400 mg moxifloxacin. The drug is clearly negative. ECG intervals were measured with a high-precision QT (HPQT) technique, and the resulting CIs are very tight. The LB of the CI for the moxifloxacin response is above 5 ms at all 4 analyzed timepoints. *Source: Shumaker et al. Poster presented at the AARC-NCI-EORTC 2011, San Francisco, November, 2011*

drug-induced changes in T-wave morphology, and the E14 Q&A document (Question 4B) therefore recommends some degree of manual oversight in terms of assessment of T-wave morphology. This is also advisable for overall signal and measurement quality control as fully automated analysis may not be completely reliable and therefore lead to higher variability of the data (Tyl et al. 2009). In this context, it is however interesting to note that automated algorithms in fact are able to *improve* the detection of subtle T-wave changes, induced by, e.g., moxifloxacin (Couderc et al. 2008).

3 Drug-Induced Effects on Other ECG Parameters

The TQT study is formally powered to exclude a small (around 5 ms) QTc prolongation. The variability of other ECG parameters (such as the PR and QRS intervals) are in fact lower than for the QTc interval, and it has become increasingly apparent that these studies also can and should be used for assessment of other ECG effects. These data, unfortunately, are not always given in publications on TQT studies, which makes it difficult to independently evaluate the QTc effect, or lack thereof. As an example, there is no mention of effects on heart rate, PR, or QRS interval in the publication on the TQT study with liraglutide (Chatterjee et al. 2009),

and no such effects are mentioned in the US prescribing information. In contrast, in the Health Canada Summary Basis of Decision, it is described that liraglutide at therapeutic doses causes a sustained increase in heart rate and prolongation of the PR interval. The incidence of subjects with heart rate values greater than 90 bpm was 20 % for 1.2 mg and 24 % for 1.8 mg liraglutide, as compared to 8 % and 4 % on the respective day for placebo. A peak placebo- and baseline-adjusted PR prolongation of 9–10 ms was seen. A PR prolongation of 7 % ms (maximum increase) was also observed in the TQT with the subcutaneous (SC) formulation of exenatide (Linnebjerg et al. 2011). The clinical relevance of these small increases in the PR interval can be debated but warrants further evaluation in terms of the incidence of high-degree AV block in late phase studies in the targeted patient population.

It seems prudent to analyze all ECG parameters in TQT studies using the same approach as for QTc, i.e., the placebo-corrected, change-from-baseline across timepoints after dosing, which also recently have been highlighted by IRT in comments on TQT study protocols.

4 ECG Assessment with Oncology Drugs

Based upon the likelihood for genotoxicity and poor tolerability, clinical ECG assessment with oncology drugs is often performed in cancer patients rather than in healthy volunteers. In this often immune-compromised and severely ill patient population, it is difficult to justify the use of placebo and an antibiotic (the positive control, moxifloxacin), and many ECG studies with oncology agents in cancer patients are therefore uncontrolled (Lesimple et al. 2013) and frequently powered to exclude a somewhat larger effect (around 20 ms) than TQT studies in healthy subjects (Bello et al. 2007; Graham et al. 2013; Rock et al. 2009; Sarapa and Britto 2008). Apart from these limitations, other elements from the TQT study design, such as strictly controlled experimental conditions, serial ECGs at baseline, and post-dosing, are implemented to the extent feasible into ECG studies in oncology patients (Rock et al. 2009).

A large number of tyrosine kinase inhibitors with indications in oncology have recently been approved or are in clinical development, and this class of drugs can illustrate the different approaches taken in terms of definitive ECG assessment (Shah et al. 2013). QT studies with these agents have been performed both in cancer patients and in healthy volunteers. Tasocitinib, a JAK 3 inhibitor in development as an oral treatment for rheumatoid arthritis (RA) and psoriasis, was evaluated in a TQT study in 60 healthy volunteers with a single supratherapeutic dose of 100 mg, placebo, and moxifloxacin. The 100 mg dose, which was estimated to generate 3.5-fold higher plasma levels than in patients on a therapeutic dose, was clearly negative, and a QTcF effect above 10 ms could be excluded at all post-dosing timepoints; the slope of the exposure-response relation was essentially flat, and the effect at the observed peak plasma level could be projected to around 0 ms (CI: −1.2 to 0.9) (Krishnaswami et al. 2011). Bosutinib, a dual Src/Abl kinase inhibitor

that targets the tyrosine kinase brc-abl, the key enzyme in the development of chronic myeloid leukemia (CML), was also tested in 60 healthy volunteers in a 2-part, single-dose, crossover, placebo-, and moxifloxacin-controlled study evaluating therapeutic and supratherapeutic exposures of the drug (Abbas et al. 2012). In a separate part of the study, supratherapeutic bosutinib plasma levels were obtained with concomitant dosing of 500 mg bosutinib (the therapeutic dose) and a strong CYP 3A4 inhibitor, ketoconazole. Since ketoconazole has a QTc effect in itself (Chaikin et al. 2005; Darpo et al. 2006; Tyl et al. 2012), the QTc effect of bosutinib in this part of the study was adjusted for ketoconazole. The UB of the 90 % CI were below 10 ms at all timepoints post-dosing for both therapeutic and supratherapeutic bosutinib plasma levels with the largest observed effect on ΔΔQTc of 4.5 ms (UB of 90 % CI: 6.8 ms) at 8 h post-dose. Lenvatinib, an orally administered tyrosine kinase inhibitor targeting VEGFR1–3, FGFR1–4, PDGFRβ, RET, and KIT, which is currently being studied in patients with solid tumors, has also been tested in healthy volunteers in a placebo-controlled study with moxifloxacin as positive control and a therapeutic dose with a negative result (Shumaker et al. 2011). Other tyrosine kinase inhibitors have only been tested in cancer patients. Sunitinib (Sutent), an oral, small-molecule, multi-targeted receptor tyrosine kinase (RTK) inhibitor approved for the treatment of renal cell carcinoma (RCC) and imatinib-resistant gastrointestinal stromal tumor (GIST), was evaluated in 24 patients with solid tumors (Bello et al. 2007), in whom QTc prolongation was demonstrated at supratherapeutic plasma levels. The largest mean ΔQTcF was 5.6 ms (UB of 90 % CI: 9.3 ms) at steady-state/therapeutic plasma levels and 15.4 ms (UB of 90 % CI: 22.4 ms) at supratherapeutic concentrations after dosing during 9 days and the ΔQTcF effect correlated with sunitinib exposure. Sorafenib (Nexavar), a bi-aryl urea inhibitor of several tyrosine protein kinases (i.e., VEGFR and PDGFR) and the C-Raf kinase approved for the treatment of advanced renal and hepatocellular carcinoma, was tested in 31 patients with advanced cancer in an uncontrolled, open-label study with a therapeutic dose of 400 mg BID (Tolcher et al. 2011). The primary endpoint in this trial was the QTc effect at each subject's Tmax at steady state (Day 1 of Cycle 2), and a mean QTc effect of 9.0 ms (SD 18 ms) was observed using this approach, whereas the time-matched effect ranged between 4.2 and 5.8 ms. Vandetanib (Caprelsa), a kinase 594 AU4 inhibitor with activity against vascular endothelial growth factor receptor 595 (VEGFR), epidermal growth factor receptor (EGFR), and the RET-tyrosine kinase, is approved for use in (metastatic) medullary thyroid cancers. The drug seems to be the most potent QT-prolonging drug among the tyrosine kinase inhibitors and was assessed in 231 patients with medullary thyroid cancer who received vandetanib 300 mg once daily in the phase 3 clinical trial (Caprelsa US NDA 022405 2011). Vandetanib was associated with a plasma concentration-dependent QTc prolongation, and based on exposure-response analysis, the mean ΔQTcF was projected to 35 ms (UB of 90 % CI: 36 ms) for the 300 mg dose (Fig. 4). Thirty-six percent of patients experienced greater than 60 ms increase in ΔQTcF, and 4.3 % of patients had QTcF greater than 500 ms, and cases of torsades de pointes and sudden death have been reported. As a result of these findings, this drug carries a black box

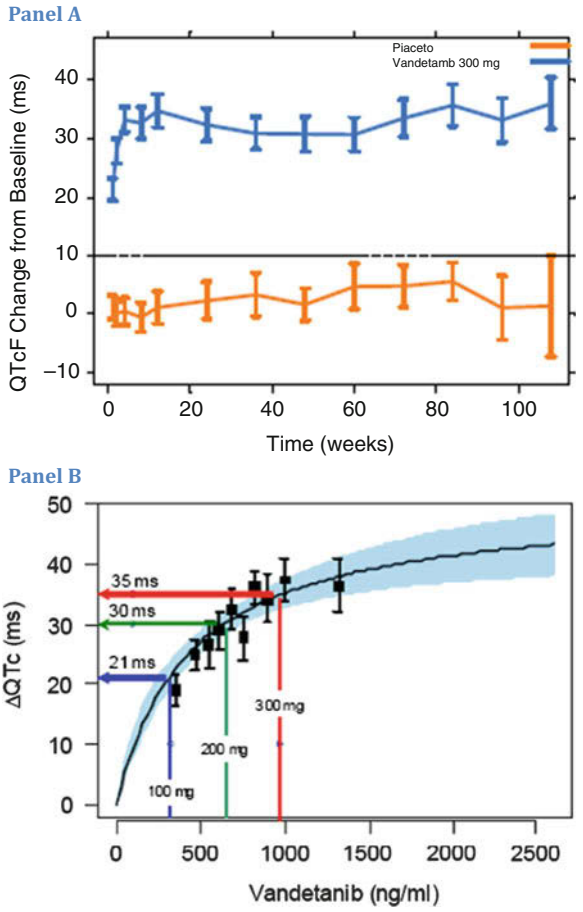


Fig. 4 QTcF prolongation in patients with medullary thyroid cancer dosed with vandetanib (Caprelsa) 300 mg daily or placebo ($n = 231$) in the pivotal trial. Panel (**a**) ΔQTcF in patients on placebo (*yellow*) and on vandetanib (*blue*). Panel (**b**) Exposure-response analysis using a mechanistic Emax model from the same patients described in Panel (**a**). The model-based estimates (*black line*) and 2-sided 90 % CI (*blue shaded area*) of ΔQTcF are shown and predicted ΔQTcF to 35 ms after daily dosing with 300 mg vandetanib and to 30 ms and 21 ms with dosing with 200 and 100 mg daily. *Source: Caprelsa US NDA 022405: Figures 3 and 4 in Clinical Pharmacology Biopharmaceutics Review, May 2011. Available at: http://www.accessdata.fda.gov/drugsatfda_docs/nda/2011/022405Orig1s000TOC.cfm*

warning for QTc prolongation, torsades de pointes, and sudden death and should not be administered with other drugs that can prolong the QTc interval.

5 Can "Early QT Assessment" Replace the TQT Study?

Exposure-response (ER) analysis has become an important tool to interpret QT data from TQT studies and has been used to predict QT effects in patients for the targeted indication, including patients with impaired clearance of the drug (Garnett et al. 2008; Piotrovsky 2005). ER analysis has also been applied to QT data derived from early SAD/MAD studies. Since the doses in SAD studies are often escalated to MTD, high plasma levels often are obtained, which allows for the evaluation of potential ECG effects over a wide range of plasma concentrations. With increasing confidence in data derived from these types of studies, the relevant question as to whether "early QT assessment" can replace the TQT study has been raised (Darpo

and Garnett 2012; Rohatagi et al. 2009; Shah and Morganroth 2012). The thorough QT (TQT) study was initially perceived as a challenge for industry (Shah 2005), but with increasing experience and refined methodologies, studies in healthy volunteers can today be conducted more effectively and with high confidence in the generated data. The TQT study is entirely designated to evaluate the ECG effects of an NCE, and the resource efficiency of this approach can be debated (Bouvy et al. 2012). A more efficient approach could therefore be to collect the same ECG data in studies that are standard components of the clinical development program, provided the same level of confidence in the generated data can be achieved. SAD/MAD studies have as their main objectives tolerability/safety and pharmacokinetics of the NCE, and when doses are pushed up to MTD levels, plasma levels above those seen during later stages of development are often reached.

5.1 The Role of Exposure-Response Relationship

Modeling of the exposure-response (ER) relationship is certainly not a new tool and has been applied to QT data before the implementation of the TQT study, especially for antiarrhythmic drugs (Allen et al. 2000; Holford et al. 1981; Phillips et al. 2001; Piergies et al. 1987; Shi et al. 2001; Whiting et al. 1980). With the application of ER to data derived from TQT studies, the role of the methodology for non-antiarrhythmic drugs has expanded to include projection of QTc prolongation with doses and formulations not directly evaluated in the TQT study and projections of QTc prolongation in specific patient populations with increased exposure to a drug and, occasionally, to help to understand ambiguous results. In the TQT study, ER analysis is performed by applying a mixed-effect model that describes the relationship of data pooled across individuals in the placebo and active groups. To account for diurnal variation, baseline-corrected QTc or placebo- and baseline-corrected QTc data are used (Florian et al. 2011; Garnett et al. 2008). For most noncardiac drugs, the relationship can be described by linear models using either observed concentrations or logarithmic-transformed concentrations. The same ER models can be applied to data obtained from SAD/MAD studies, from which the time-matched concentration and baseline-corrected QTc data can be pooled across placebo and active cohorts. Figures 5 and 6 show two examples, one negative and one positive, from ER analysis applied to data derived from SAD/MAD studies. The first example illustrates that ER modeling applied to data derived from a typical SAD study with a carefully designed ECG schedule can, despite the small sample size in each dose group, achieve sufficient power to exclude a QTc effect exceeding the regulatory concern, i.e., 10 ms. The second example derives from a MAD study, which clearly demonstrated a QT-prolonging effect of the NCE; for every 100 ng/mL increase in plasma concentration, the QTc interval can be projected to increase by 1.9 (90 % CI: $\pm$ 0.45) ms and the UB of the CI clearly exceeded the 10 ms threshold. In the latter example, given the precision of the predicted QTc effect with a statistically positive slope, it seems highly likely that a subsequent TQT study would derive the same conclusion and the study can therefore be avoided, since the

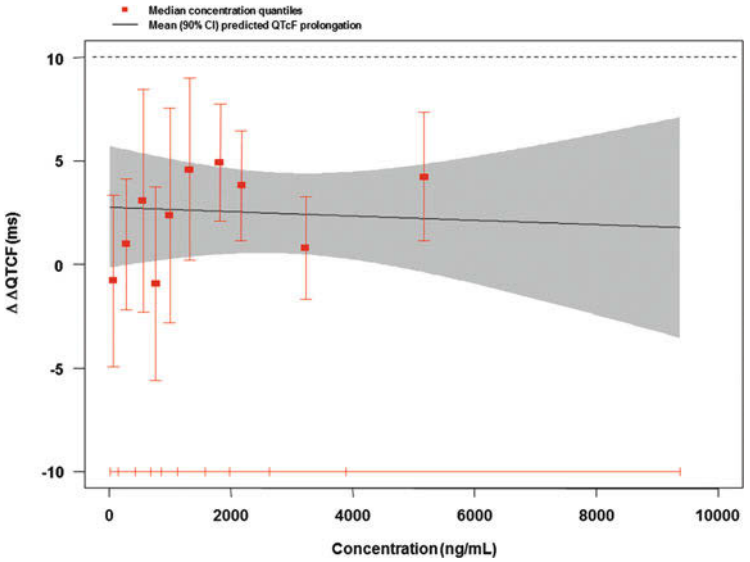


Fig. 5 Data from a standard design single-ascending dose (SAD) clinical study with 6 subjects on active treatment and two subjects on placebo in each dosing group. A total of 7 doses were studied with a 120-fold range between the lowest and the highest tested dose, resulting in 42 subjects on active treatment and 14 on placebo. Continuous 12-lead ECGs were recorded; ECGs were extracted and PK samples collected at prespecified timepoints at baseline and post-dosing. QT intervals were measured using a high-precision QT measurement (HPQT) technique on all beats from 10 replicates at each timepoint (Darpo et al. 2011). The figure shows the prediction of ΔΔQTcF across the plasma concentration range observed in the study with model-based estimates (*black line*) and 2-sided 90 % confidence interval (CI; *gray-shaded area*) of the QTc effect. The slope of the ER relationship was slightly negative and not statistically significant (−0.00026 ms per ng/mL; 90 % CI: −0.00063 to 0.00010). The upper bound of the CI was clearly below 10 ms at all concentrations observed in the study, meaning that the drug did not cause QT prolongation exceeding the threshold of concern. The average variability of the QTc estimate over all timepoints, measured as the between-subject SD of ΔQTcF, was very low, 6 ms. *Reproduced from Darpo and Garnett (2012) with permission from Br J Clin Pharmacology, The British Pharmacological Society and Blackwell Publishing*

consequence for further development of the compound would be the same if the benefit/risk assessment remained favorable: the QTc effect must be further characterized in the targeted patient population. This example therefore illustrates how a QTc effect can be detected using ER modeling of data derived from a MAD study and thereby potentially waive the need for a TQT study (Stockbridge et al. 2012). It should be acknowledged that the QTc effect in the example was large and detection or exclusion of smaller effect levels will pose more of a challenge and will require high standards for the clinical conduct and QT interval measurements.

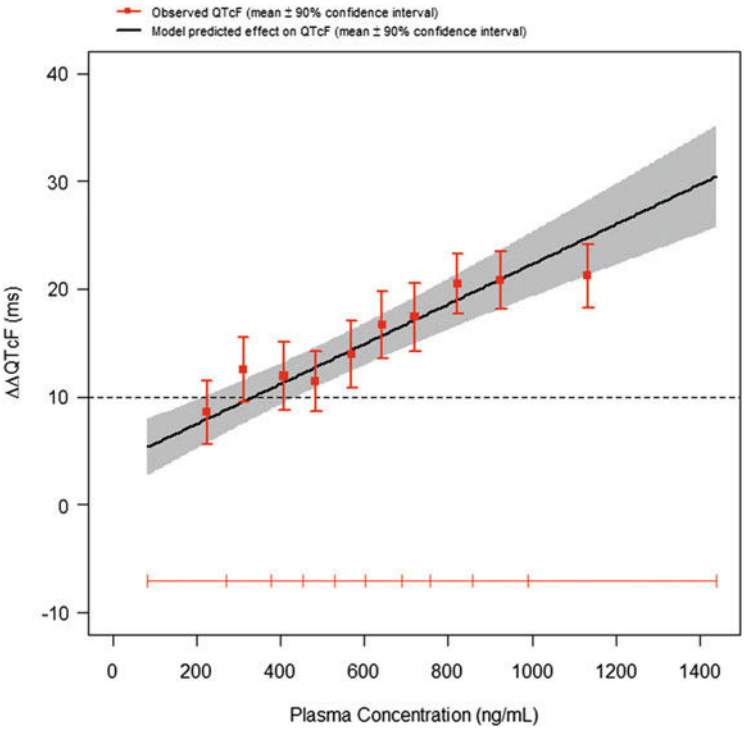


Fig. 6 Data from a MAD study in which subjects received either placebo or 2 dose levels of the NCE in a fixed sequence, resulting in 8 subjects on the lowest dose and 16 subjects on the 2 higher doses and on placebo. ECGs were extracted in 10 replicates from continuous 12-lead recordings at baseline (predose) on Day 1 and at 7 timepoints on Day 7, paired with PK samples. ECG intervals were measured using the same high-precision QT measurement (HPQT) methodology as in this figure, and observed between-subject standard deviation of ΔQTcF was 7 ms on active and 8 ms on placebo. A concentration-dependent effect of the drug on ΔΔQTcF was demonstrated with a statistically significant slope of 0.0185 ms per ng/mL (CI: 0.014 to 0.023, $p < 0.0001$). *Reproduced from Darpo and Garnett (2012) with permission from Br J Clin Pharmacology, The British Pharmacological Society and Blackwell Publishing*

5.2 The Power of Exposure-Response Analysis Compared to the E14 Time-Matched Analyses

The ICH E14 guidance requests that data are analyzed "by timepoint," which means that a QTc effect (measured as the placebo-adjusted change-from-baseline; ΔΔQTc) exceeding 10 ms must be excluded at each post-dosing timepoint irrespective of the observed plasma levels of the drug. Formal power calculation using an assumption of SD of ΔQTc of 10–12 ms, which is the QTc variability typically observed with a semiautomated QT measurement techniques (see Table 1), shows that 60–90 subjects are required to achieve acceptable power (90 %) to exclude a 10 ms effect (Tsong et al. 2010). For standard SAD/MAD studies with

sample sizes of around 6–8 subjects per dose group, the power of a time-matched analysis is therefore insufficient. In contrast, when using ER analysis, all observed QTc intervals at given plasma levels of the drug are analyzed in one model, and this approach therefore provides a higher power to detect or exclude a QTc effect. In a simulation study based on moxifloxacin and placebo data from 5 crossover-designed TQT studies, different underlying QTc effect levels (no effect, 3 ms, and 5 ms) were simulated, and the power to exclude a QTc effect was compared between the E14 time-matched (TM) analysis and ER-response analysis using 1,000 and 3,000 resamples of the data (Ferber 2012). When a small underlying effect of 3 ms was simulated, ER provided 76–99 % power to exclude a 10 ms effect with 9 subjects, whereas the power of the TM approach was too low (26–67 %). Likewise, if no underlying effect was simulated, the power of ER to exclude an effect above 10 ms was 92–100 % with 9 subjects and that of TM was 43–87 %. Even though these numbers should be confirmed using additional data sets and statistical approaches, the simulation exercise clearly confirms that ER analysis provides much higher power than the E14 time-matched approach and can therefore more effectively be used in SAD/MAD studies of typical size.

5.3 How Can Assay Sensitivity Be Demonstrated Without Using a Pharmacological Positive Control?

It seems unjustified and undesirable to use moxifloxacin or any other pharmacological positive control in SAD/MAD studies, and alternative methods must therefore be sought to confirm that the study is sufficiently sensitive to detect or exclude a small QT effect, i.e., to demonstrate assay sensitivity. The inclusion of moxifloxacin as a positive control in TQT studies has been a key factor for achieving a high confidence in the study's ability to demonstrate the absence of a drug effect, and it can be assumed that some method to demonstrate assay sensitivity will be needed if SAD/MAD studies are to replace the TQT study. It also may seem unlikely that a concept with "accredited" clinical study centers and central ECG laboratories will be implemented since this will not provide assurance that studies conducted some time apart will all have the same ability to exclude small QT effects based on various factors, such as staff turnover, differences across study populations, or just inherent variability of the data. A more realistic approach is to use data derived in each study to confirm that the study held sufficient quality to detect a small drug effect, even though there was none with the studied NCE. Recently published research supports this approach and suggests that study-specific quality criteria may replace the positive control (Malik et al. 2011). The within- and between-subject variability across several, complete baseline days from several TQT studies was evaluated, and proposed statistical analyses seem to differentiate between studies of high and poor quality. Small changes related to diurnal variability are certainly not the same as small drug-induced changes, but it seems appropriate that tests of "change" irrespective of the underlying mechanisms can provide the necessary assurance of assay sensitivity.

Given the small sample size of a typical SAD study, the risk of a false-negative result (the study fails to demonstrate a QT effect when there is one) is substantially larger than the risk of a false-positive one (inability to exclude a small QT effect when there is none). Given this imbalance, it may be that the requirement for demonstrating "assay sensitivity" eventually becomes less prescriptive as experience with "early QT assessment" accumulates.

6 The Path Forward Toward Replacing the TQT Study

Almost 8 years have now elapsed since the implementation of the ICH E14 guidance in May 2005, and several hundreds of TQT studies that basically follow the E14 guidance have been performed and submitted to regulatory authorities. As of October 2012, the FDA's IRT had evaluated 288 TQT studies. Based on the high confidence in data derived from the TQT study, it will be challenging to replace it with "early QT assessment," and the process, if successful, will likely include several steps. Generation of more prospective data to demonstrate that "early QT assessment" can provide results concordant to the results of TQT studies will be required, and alternative methods for demonstrating assay sensitivity will have to be successfully tested. It is the E14 "threshold of concern" ($<$10 ms) on which the confidence that a drug with a negative TQT study is truly devoid of proarrhythmic liability in patients is based. However difficult to prove, it is generally accepted that the TQT study has been very effective in terms of protecting patients by identifying "QT liability" for new drugs (Stockbridge et al. 2012), with consequent regulatory actions (precautionary statements, black box warnings, restricted access and withdrawals). It seems highly unlikely that a different threshold will be widely accepted across regions without substantial further advancement of our knowledge of the relationship between mild QT prolongation and its consequences in large populations. The same threshold should therefore be used for "early QT assessment" based on ER analysis, i.e., the upper bound of the 2-sided 90 % CI of the QTc estimate should be lower than 10 ms at concentrations that are relevantly high for the targeted patient population. The TQT study will not be replaced with "early QT assessment" overnight, and it seems unlikely that ICH E14 will be revised until a sufficient amount of data have convinced all participating parties that alternative approaches can provide data at the same level of confidence as the TQT study. Replacing the TQT study will therefore probably be a stepwise, staggered approach, in which the request for a TQT study may be waived for some compounds with certain characteristics, while others will have to undergo a TQT study. Examples of the former may include compounds from a pharmacological class known to have no members with QT liability, a clean nonclinical safety pharmacology package, and robustly negative ER analysis of SAD/MAD data with the upper bound of the 2-sided 90 % CI of the projected QTc effect below 10 ms at concentrations that are relevant for the targeted patient population. Other drugs, such as those with a small underlying effect or where "early QT assessment"

has not provided a sufficiently precise estimate of the QT effect, would still require an E14-compliant TQT study.

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Index

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