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New Approaches to Drug Discovery

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Preface

The last decade has seen many exciting new medicines being introduced to the market, such as novel oral anticoagulants, novel anti-diabetics, highly effective antiviral agents against hepatitis C, oral MS therapies, or targeted cancer therapies to name just a few. For the first time, diseases with orphan drug designations, e.g., cystic fibrosis or rare blood disorders, are treated with new chemical or biological entities. Biologics have now reached center stage especially for the treatment of immune disorders and oncologic indications, with the anti-TNFα agent adalimumab being the best-selling drug in 2014. These impressive successes notwithstanding, the so-called patent cliff, a conceived lack of productivity in the pharmaceutical industry, increasing expenses to discover and develop new therapeutic agents and reimbursement challenges have put pressure on the community to only target highly innovative approaches and to focus resources in selected areas of high expertise. With increased investments over the last years pharma companies continue to support large R&D efforts, while new venture capital backed biotech companies have surfaced, and universities attempt to translate their basic research into products through collaborations and the build-up of screening centers. Apart from this dynamic, the underlying process of drug discovery has not changed dramatically. It still starts with a solid disease hypothesis linked to a target which then needs precise validation (D. Sim, K. Kauser, B. Nicke) before the high-throughput screening is started for lead identification. As pointed out by J. Eder and P.L. Herrling, phenotypic screens have gained more attention recently, where a cell-based assay is used to first identify leads and later – hopefully – the corresponding target. Intractable targets, discarded as non-druggable a decade ago, are tackled today (S. Knapp), and new chemical matter intercepting protein–protein interactions (C. Ottmann), a revived interest in natural products (E.F. van Herwerden, R. Süssmuth), and powerful high-throughput synthesis (C. Rademacher, P.H. Seeberger) might help to dissect and address challenging pathways. Meanwhile classical medicinal chemistry can rely on improved predictive models (M.S. Lawless, M. Waldman, R. Fraczkiewicz, R.D. Clark) and strong in vitro assays (G. Langer) to identify and optimize leads. Understanding their pharmacokinetic properties (A. Reichel) is a prerequisite for lead refinement and candidate selection, before in vivo efficacy is demonstrated in relevant animal models (O.D. Slayden, H. Trübel, B. Albrecht, J. Hoffmann) and the potential

candidates are subjected to a thorough safety assessments (C. Stark) for final triaging. Early identification of biomarkers to either select patients susceptible to a certain therapy or as surrogate marker for efficacy (T. Krahn) and computational models to simulate drug effects (J. Lippert) have become essential tools when entering the clinical phase.

We hope the handbook conveys the excitement and progress made in drug discovery throughout the last decade. While the process has stayed the same, it has been enriched and reinvented along the value chain. Hence, Giuseppe di Lampedusa's *Se vogliamo che tutto rimanga come è, bisogna che tutto cambi* [If we want things to stay as they are, things will have to change, Il Gattopardo (1958)] might probably be an appropriate motto for this book.

Berlin, Germany

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Trends in Modern Drug Discovery

Jörg Eder and Paul L. Herrling

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Abstract

Drugs discovered by the pharmaceutical industry over the past 100 years have dramatically changed the practice of medicine and impacted on many aspects of our culture. For many years, drug discovery was a target- and mechanism-

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agnostic approach that was based on ethnobotanical knowledge often fueled by serendipity. With the advent of modern molecular biology methods and based on knowledge of the human genome, drug discovery has now largely changed into a hypothesis-driven target-based approach, a development which was paralleled by significant environmental changes in the pharmaceutical industry. Laboratories became increasingly computerized and automated, and geographically dispersed research sites are now more and more clustered into large centers to capture technological and biological synergies. Today, academia, the regulatory agencies, and the pharmaceutical industry all contribute to drug discovery, and, in order to translate the basic science into new medical treatments for unmet medical needs, pharmaceutical companies have to have a critical mass of excellent scientists working in many therapeutic fields, disciplines, and technologies. The imperative for the pharmaceutical industry to discover breakthrough medicines is matched by the increasing numbers of first-in-class drugs approved in recent years and reflects the impact of modern drug discovery approaches, technologies, and genomics.

Keywords
Pharmaceutical research · Pharmaceutical industry · R&D productivity · Target-based drug discovery · Phenotypic screening · Lead discovery · Target discovery

1 The Beginnings of Modern Drug Discovery

Modern drug discovery is one of the most complex scientific areas and involves many different scientific disciplines. It has its origins at the end of the nineteenth century in the experimental biological and medical research of Claude Bernard, Louis Pasteur, Robert Koch, Paul Ehrlich, and Joseph Lister, as well as in the great advances in organic chemistry at the same time, and has ever since dramatically changed the practice of medicine, our culture, and sociology. About 1,500 unique drugs are currently known which act through more than 350 different mechanisms (Overington et al. 2006). With these, many diseases are now curable or can at least be controlled at the symptomatic level including bacterial, parasitic and viral infections, rheumatoid arthritis, asthma, osteoporosis, thrombosis and other cardiovascular disorders, diabetes, psychiatric diseases, and various cancers. Moreover, drugs have enabled many surgical procedures of modern medicine and even made cell and solid organ transplantation possible.

The first 100 years of modern drug discovery were largely target and mechanism agnostic and primarily driven by chemocentric approaches, i.e., approaches based on a specific compound or compound class which served as starting point for further optimization. These chemotypes were either discovered through ethnobotanical knowledge or derived from natural ligands and substances. Serendipity, however, was also an important success factor in many instances. In the following we list a few examples to illustrate how drugs were discovered during this time period.

1.1 Aspirin

Extracts of the bark from the willow tree were used for thousands of years in Europe and North America for pain relief, treatment of inflammation, and fever. The active ingredient of the bark extract was first isolated by the German chemist Johann Andreas Buchner in 1828 and named salicin after the Latin name for the white willow (*Salix alba*). The glycoside can be converted to salicylic acid by hydrolysis and subsequent oxidation (Fig. 1). Felix Hoffman, a chemist at Bayer in Germany,

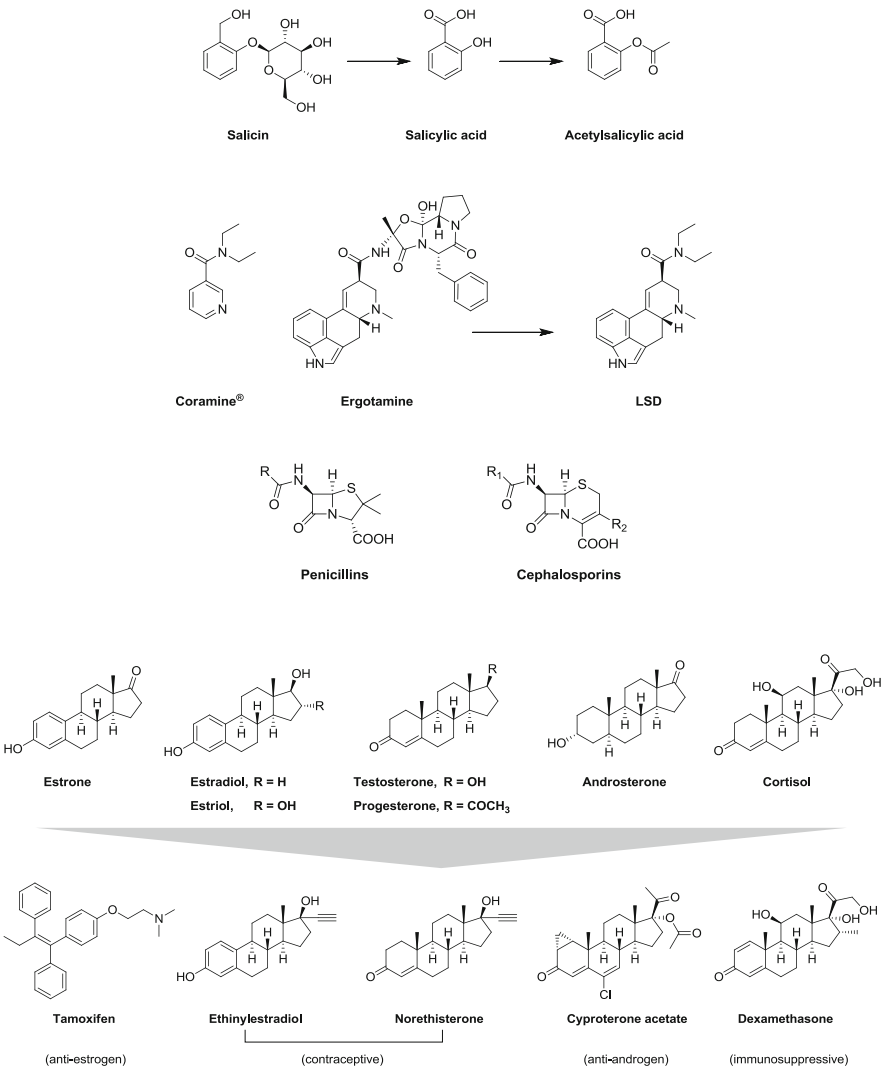

Fig. 1 Drugs discovered during the first 100 years of modern drug discovery were mainly based on ethnobotanical knowledge or derived from natural ligands and substances

systematically searched for derivatives of salicylic acid in 1897. His search was triggered by his father who suffered from rheumatoid arthritis and did not tolerate high doses of salicylic acid due to intestinal tract irritation and emesis. One of the first derivatives he synthesized was acetylsalicylic acid which is known since 1899 as Aspirin® (Schrör 2008). It took more than 75 years until it was discovered that the chemical derivatization also led to an advantageous change in the mechanism of action as it turns the drug into an irreversible inhibitor thereby preserving its therapeutic effect beyond compound exposure. The latter is the basis for the success of low-dose acetylsalicylic acid treatment as anticoagulant therapy used by millions of patients today.

1.2 Ergotamine

The fungus *Claviceps purpurea* and other clavicipitaleans form ergot sclerotia to produce their spores in oat, rye, wheat, and other grasses. These sclerotia contain more than 50 different indole alkaloids, referred to as ergot alkaloids. Many of these are highly toxic due to their vasoconstrictive properties leading to gangrenous loss of the limbs, hallucinations, and dementia. The first documented ergotism epidemic of human toxicity occurred in central Europe in 857 AD, and it took almost 1,000 years to realize the causal relationship and improve agricultural practices. In herbal medicine ergot was first mentioned in the late sixteenth century for use in obstetrics to induce uterine contractions and hasten childbirth, to reduce postpartum hemorrhage, and to induce abortion. When migraine was proposed to be caused by vasodilation by sympathetic deficit in the mid-nineteenth century, the British surgeon Edward Woakes recommended ergot as a vasoconstricting treatment in 1868 (Woakes 1868). The Swiss biochemist Arthur Stoll isolated ergotamine as the active ingredient of ergot sclerotia in 1918 at Sandoz, and the company started to market ergotamine (Gynergen®) in 1921 for the treatment of migraine. Twenty years later, Albert Hofmann, a chemist and coworker of Arthur Stoll who worked on the isolation and synthesis of active ergot constituents, wanted to engraft the respiratory and circulatory stimulating effect of nicotinic acid diethylamide (marketed under the trade name of Coramine®) onto the ergotamine structure. The result was the discovery of lysergic acid diethylamide, better known as LSD, a psychedelic drug (Fig. 1).

1.3 Penicillin

Alexander Fleming serendipitously discovered the antibiotic effect of the fungus *Penicillium rubens* in 1928 when working with staphylococci cultures (Fleming 1929). One such culture was contaminated with a fungus, and the colonies of staphylococci around the mold were destroyed, whereas other colonies farther away were unaffected. He grew the fungus in pure culture, established that it also killed other disease-causing bacteria, and named the unknown active ingredient

penicillin. Only 12 years later the pure substance was isolated and characterized and its chemical structure determined (Fig. 1). The success of penicillin and its derivatives triggered a search for additional antibiotics produced by other fungi and led, for example, to the discovery of cephalosporins (Fig. 1) which have the same mechanism of action but are less prone to hydrolysis by bacterial β-lactamases.

1.4 Steroid Hormones

The first steroid hormone was isolated from the urine of pregnant women by Adolf Butenandt in 1929 (estrone; see Fig. 1) (Butenandt 1931). To guide the isolation, he used a specific test system to detect the activity of the hormone. In the following years, he and others isolated and structurally characterized other female (estradiol, estriol) and male (testosterone, androsterone) sex hormones, progestogens (progesterone), and corticosteroids (e.g., cortisol). Their chemical optimization toward oral bioavailability and the search for more potent analogs led to a number of important drugs in the field of cancer (antiestrogens, antiandrogens; see Fig. 1) and immune diseases (e.g., dexamethasone). In addition, the idea to combine an estrogen and progestogen by Carl Djerassi and Gregory Pincus in the 1950s gave rise to the first oral contraceptive pill and revolutionized family planning in the industrialized world.

Until modern molecular biology techniques were established in the mid-1980s, the molecular basis of the pharmacology of most drugs was not known. Pharmacological receptors, a concept proposed by Langley (1905), were only a model inferred from dose-response curves derived from measuring the effect of pharmacological agents applied to whole animals or isolated tissues, such as the muscle, gut, and heart in organ bath apparatuses (Fig. 2). This was still the case in 1975 (Goodman and Gilman 1975). It was assumed already in 1880 by Langley (1880)

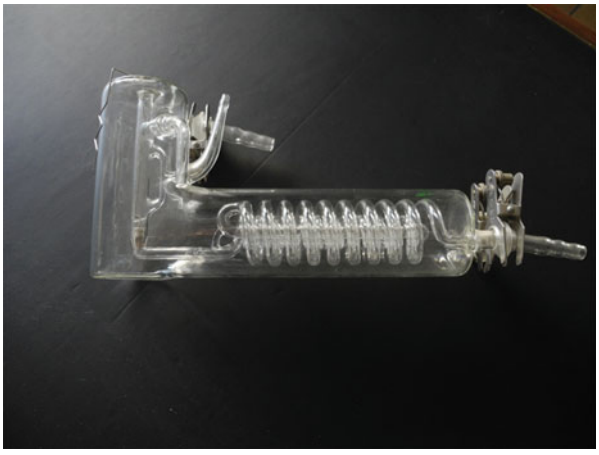

Fig. 2 Organ bath used in the author's laboratory (PH) in the 1980s

that actions of pharmaceutical drugs are governed by the law of mass action. This concept was further elaborated by Clark (1920). The receptor existed only as an abstract model, but its interactions with pharmacological drugs could be measured by constructing logarithmic dose-response curves as well as the interactions of agonists and antagonists at a particular receptor (Kenakin 1987; Arunlakshna and Schild 1959). The availability of radioactive selective receptor ligands and the development of receptor binding studies by Robert Lefkowitz et al. (1970) have greatly helped to localize receptors in different organs and tissues in particular in the brain (Cortes et al. 1987) as one example of many.

Meanwhile, due to advances in molecular biology, genetics, protein sequencing, and computing, many receptors and other drug targets are cloned, purified, and described atom by atom in spatial models allowing true target-based drug discovery, i.e., studying the interactions of targets with drug molecules in isolation and visualizing and calculating their interactions at atomic scale (Falchi et al. 2014; Chen et al. 2012).

2 Where Do Chemical Lead Structures Come from Today?

Target-based drug discovery has enabled a great expansion of chemotypes and pharmacophores available for the medicinal chemist during the past three decades. New techniques like high-throughput screening (HTS), fragment-based screening (FBS), crystallography in combination with molecular modeling, and combinatorial and parallel chemistry have created a considerable diversity of chemical lead structures well beyond the known natural products and ligands used as chemical starting points for drug discovery in the past. Moreover, this wealth of chemotypes can now be used as a source for tool compounds to study unexplored biological space and find new drug targets or for phenotypic screening using systems-based approaches to identify drug candidates in a target-agnostic manner (see below). Figure 3 shows examples of successful target-based drug discovery projects using the different methods available for identification of lead structures. These include high-throughput screening of diverse chemical libraries, fragment-based screening, rational drug design, the use of target family knowledge, and in silico drug discovery methods.

2.1 Origin of Libraries

Typically, the libraries are composed of the compounds synthesized over time by individual companies and influenced by a company's history, e.g., Novartis has a large number of ergot compounds in its library, and Roche would have many benzodiazepines. But as many companies work on similar targets or scaffolds, there must also be some overlap between the libraries. Nevertheless these libraries are a key component of the success of pharmaceutical companies, although they have once been in danger of getting lost. At the time combinatorial chemistry

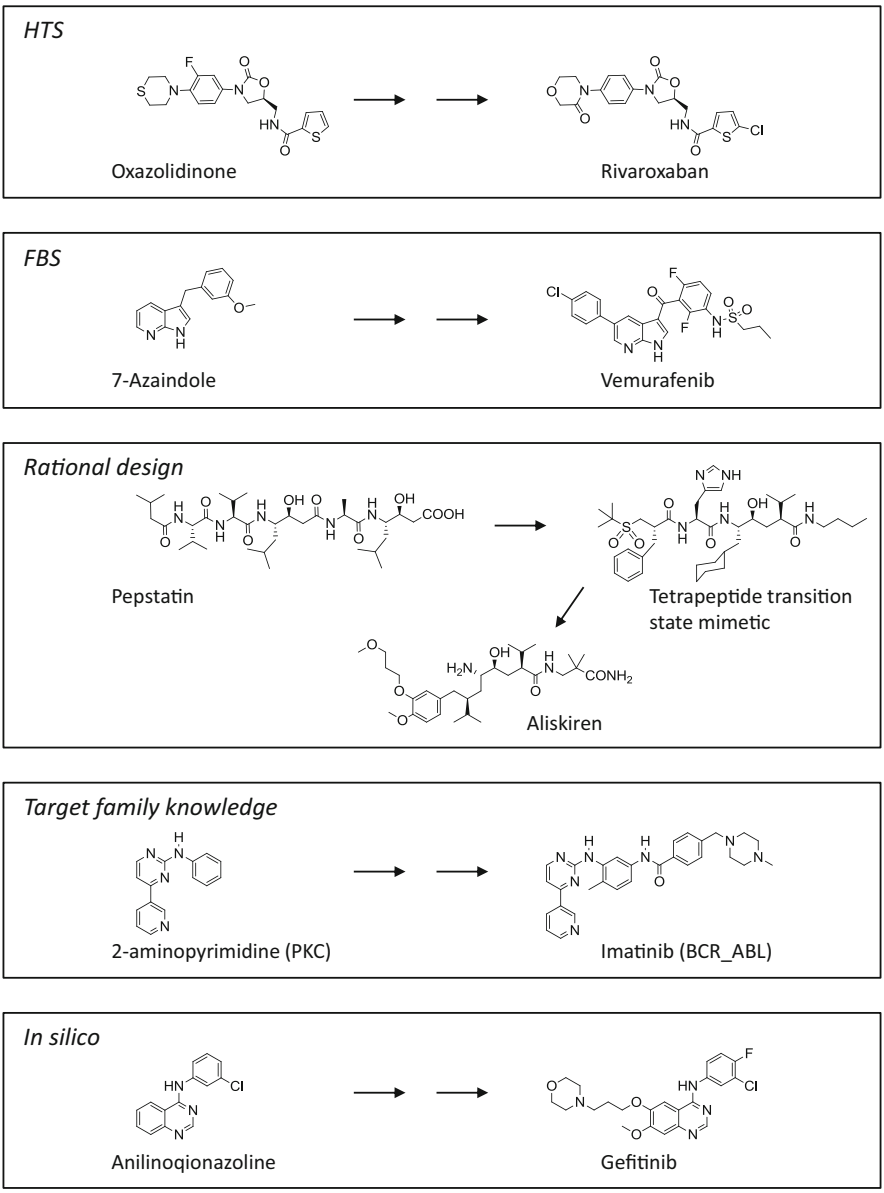

Fig. 3 Target-based drug discovery has enabled a great expansion of pharmacophores by using a variety of different methods

became possible in the 1980s eventually allowing the rapid synthesis of millions of compounds it was thought that all possible compounds could be made when needed by starting from individual scaffolds, and the historical libraries were neglected for

a while. However, it became apparent in HTS that the hit rate, when using these combinatorial libraries, was distinctly lower than with the historical libraries (Lahana 1999). One reason for this was that combinatorial libraries were strongly dependent on chemical parameters, such as the possibility to do chemistry with molecules attached on beads rather than on potential biological activity alone. This insight led to a revalorization of the collection of historical compounds that had been made for pharmacological activity. It also led some companies to maintain and expand their natural-compound libraries as these can be seen as compounds selected for biological activity for hundreds of millions of years. Medically useful compounds from natural substances are described above. Today the realization that even the millions of compounds available cover only a small part of the biologically active compound universe makes it important to continue the efforts to diversify our libraries as repeatedly few or no ligands are found in the existing libraries for some newly discovered targets.

2.2 HTS

Compound collections used for high-throughput screening are typically based on chemically diverse molecules as well as on chemotypes from previous projects and can reach a size of 1–2 million substances. The compounds are screened in biological test systems, and hits, once validated by independent biochemical or biophysical methods, are further optimized to drug candidates. An example is the discovery of the anticoagulant rivaroxaban, a factor Xa inhibitor approved by the FDA in 2011. The HTS hit selected for further optimization was an oxazolidinone derivative (Perzborn et al. 2011), a compound class previously worked on for inhibition of the 50S ribosomal subunit A site in bacteria.

2.3 FBS

A specific variant of HTS is fragment-based screening. It is based on the idea that smaller molecules (usually with molecular weights below 250 Da) are better suited to sample the chemical space because it is much less complex for small molecules than it is for bigger ones. Hits are generally more frequent but may only bind weakly to the biological target, which requires growing them or combining them to produce a lead with a high affinity. So far the only successful example of this relatively new technology is the BRAF V600E mutant kinase inhibitor vemurafenib. The underlying chemotype was discovered by FBS using a panel of recombinant kinases (Tsai et al. 2008). The 7-azaindole compound was subsequently optimized to the final inhibitor by conventional medicinal chemistry methods.

2.4 Rational Drug Design

The renin inhibitor aliskiren has been approved for treatment of hypertension in 2007. Renin is an aspartic protease which catalyzes the rate-limiting step in the renin-angiotensin system. Aliskiren is the product of rational drug design utilizing the inhibitory principle of pepstatin, a naturally occurring hexa-peptide which contains the unusual γ-amino acid statin. The statin-based inhibitory principle was grafted onto small peptide-like compounds derived from the natural renin substrate, and these compounds were further optimized to the final drug using structural information (Maibaum and Feldman 2009).

2.5 Target Family Knowledge

Leveraging target family knowledge is another way of generating chemical starting points for targets which are members of larger protein families such as kinases, proteases, E3 ligases, or G-protein-coupled receptors. The BCR-ABL kinase inhibitor imatinib, which revolutionized the treatment of chronic myelogenous leukemia (CML), was discovered based on an aminopyrimidine lead compound that was originally identified in a screen for inhibitors of protein kinase C (Capdeville et al. 2002). Chemical optimization toward BCR-ABL selectivity and oral bioavailability led to the final molecule.

2.6 In Silico Methods

The availability of three-dimensional structures and ever more sophisticated computer modeling programs also enables the in silico discovery of chemical starting points. An example is gefitinib, an epidermal growth factor receptor tyrosine kinase inhibitor for the treatment of lung and breast cancers. The proposed catalytic mechanism was used to define a query for structure-based searches which led to the discovery of anilinoquinazolines as potent inhibitors and suitable lead structure for this enzyme (Ward et al. 1994).

Today, in many cases, the X-ray crystal structure of a target is available early during a drug discovery project, and even the structures of membrane receptors can now be solved. With this structural information, it is often possible to combine the different lead-finding approaches into a broader, integrated lead-finding strategy. The structural information gained from each individual hit thereby adds to an overall understanding of how best to fill the binding pocket of a target and can be used to design new chemotypes based on a holistic understanding of the contributions of many diverse molecular substructures. Different to previous times, individual compound classes thereby no longer serve as separate and unconnected starting points for the medicinal chemist but contribute to an integrated strategy. Moreover, hit finding, in particular FBS, can be used to exhaustively map a target's binding site and provide the chemists and molecular modelers with

valuable ideas for the design of new chemotypes and, perhaps even more importantly, for the further optimization of lead structures. In this way, lead finding today may no longer be seen as a one-off activity at the beginning of a drug discovery project but rather as a continuing activity which accompanies compound optimization. Both, lead finding and lead optimization, can cross-fertilize each other, and the former may be run in iterative cycles with knowledge gained from previous cycles as well as lead optimization efforts feeding forward into the next cycle.

2.7 Biologics

Modern molecular biology techniques have also expanded the drug space beyond traditional synthetic small molecular weight compounds and have enabled the design, production, and development of biologic molecules as drugs. Of the 624 drugs approved by the FDA over the past 20 years, 84 were biologics (Mullard 2014). However, their impact for the pharmaceutical industry has been even bigger than these numbers suggest as seven of the ten biggest selling drugs in 2013 were biologics. So far these drugs were dominated by antibodies, soluble receptor constructs, immunoglobulin fusion proteins, and secreted naturally occurring proteins. The most prominent examples are tumor necrosis factor (TNF) alpha-blocking antibodies (infliximab, adalimumab) and the soluble TNF receptor fusion protein (etanercept) for the treatment of rheumatoid arthritis, the anti-CD20 antibody rituximab for non-Hodgkin's lymphoma, the anti-vascular endothelial growth factor A (VEGF-A) antibody bevacizumab for colorectal and other cancers, and the antihuman epidermal growth factor receptor 2 (HER2) antibody trastuzumab for the treatment of breast cancer. Beyond these "classical" drugs, the biologics space has grown over recent years, for example, by introduction of antibody-small molecular weight drug conjugates or bispecific antibodies, and is likely to continue to grow at a rapid pace over the coming years. The advantages of biologics are their high affinity for and specificity to their targets, but so far they are mostly limited to secreted or cell surface targets.

3 Where Do Targets Come from?

A minority of drug discovery projects prior to the mid-1980s were target based. One such case is the discovery of statins as HMG-CoA reductase inhibitors to lower cholesterol levels (Tobert 2003). Details of the cholesterol biosynthesis pathway were worked out in the 1950s and 1960s and HMG-CoA reductase established as the rate-limiting enzyme. The first potent inhibitor was found in the mid-1970s using an assay that involved radioactively labeled substrates in cell extracts. Today the establishment of targets for drug discovery is in many cases still based on advances in basic science over many decades and constituted by a series of important discoveries. For example, the capacity of tumor cells to stimulate angiogenesis was discovered in 1945 (Algire and Chalkley 1945) and the presence of soluble tumor-derived factors demonstrated in 1968 (Greenblatt and Shubi 1968).

This led to the formulation of the "antiangiogenesis" therapeutic concept for treatment of tumors (Folkman 1971). The subsequent purification of VEGF-A and its cloning in 1989 (Leung et al. 1989) facilitated the discovery of bevacizumab, the first anti-VEGF-A antibody (Presta et al. 1997). Another example is the discovery of imatinib for the treatment of chronic myelogenous leukemia (CML) (Capdeville et al. 2002). A chromosomal abnormality, the "Philadelphia chromosome," was discovered in 1960 in white blood cells of patients with CML. In 1973 the Philadelphia chromosome was shown to be a translocation between chromosomes 9 and 22. A series of subsequent discoveries resulted 1985 in the insight that the chromosomal translocation leads to the expression of the BCR-Abl fusion protein and the hypothesis that its tyrosine kinase activity drives malignant transformation (Shtivelman et al. 1985). Imatinib was subsequently developed as an inhibitor of the BCR-Abl kinase. The pace in the advancement of such fundamental science for the discovery of drug targets has dramatically increased with the sequencing of the human genome and the establishment of next-generation sequencing technologies. Many recently approved drugs, in particular in the oncology field, are targeting proteins that have been identified through human genetic information. This includes the discovery of ibrutinib, an inhibitor of Bruton's tyrosine kinase for the treatment of B-cell lymphomas (Honigberg et al. 2010); vemurafenib, an inhibitor of the activating mutant $BRAF^{V600E}$ protein for melanoma (Sala et al. 2008); and the Janus kinase 1 and 2 inhibitor ruxolitinib for myeloproliferative neoplasms (Quintás-Cardama et al. 2010).

Pharmaceutical or small molecular weight tool compounds have similarly helped to study complex biological systems and allowed the identification and characterization of novel drug targets. One of many examples is the discovery and validation of phosphodiesterase four isoenzymes for the treatment of lung diseases using nonspecific and isoenzyme-specific inhibitors (Torphy and Undem 1991). This ultimately led to the discovery of roflumilast for the treatment of chronic obstructive pulmonary disease. Over the past decades, these pharmacological tools were more and more complemented with biological tools, in particular antibodies, to study the functional roles of secreted proteins and receptors in vitro and in vivo. Many of these biological tools were directly developed as therapeutics once the target characterization and validation studies proofed promising. A showcase is cytotoxic T-lymphocyte-associated protein-4 (CTLA-4), a member of the immunoglobulin superfamily, which is expressed on the surface of T cells and transmits an inhibitory signal to these cells. The relevant scientific findings that define this target were made using specific monoclonal antibodies which block the binding to its ligands CD80 and CD86 on antigen-presenting cells thus leading to T-cell activation (Linsley et al. 1992) as well as with a CTLA4-IgG Fc fusion protein which binds to CD80 and CD86 and prevents T-cell activation (Linsley et al. 1991). The former has been developed as therapeutic for cancer immunotherapy (ipilimumab) and the latter for the treatment of rheumatoid arthritis (abatacept). Other important biological tools today are based on interference RNA (RNA_i) (Mohr et al. 2014) and CRISPR (clustered regularly interspaced short palindromic repeats) (Doudna and Charpentier 2014) technologies which allow specific gene expression silencing or even enable surgical genome editing, respectively.

Such tools can be used for both dedicated reverse genetic experiments and broad, even genome-wide, screens.

Today, the existence of large and diverse compound libraries in combination with great advances in cell and organoid culture technologies makes phenotypic screening also an interesting approach for target and drug discovery. The recently approved hepatitis C virus NS5A inhibitor ledipasvir is based on the discovery of the target as well as the chemical lead structure in a phenotypic screen using a viral replicon system in a human hepatocyte cell line (Gao et al. 2010). Such screens can be extended to whole organisms. The first-in-class antimalarial drug KAE609 currently in phase 2 clinical trials was discovered employing a *Plasmodium* whole-cell proliferation assay with cultured intraerythrocytic parasites (Rottmann et al. 2010). KAE609 is a spiroindolone that targets the P-type cation-transporter ATPase4, a membrane transporter protein regulating sodium homeostasis and thus the osmoregularity of the parasite. Like artemisinin, KAE609 targets all stages of the life cycle of malaria parasites which is important for fast parasite clearance. Interestingly, phenotypic screening appears to be particularly successful for antiparasitic drugs, and around 80% of new antimalarial drugs in preclinical or early clinical phases at the moment have come from phenotypic screens (Cully 2014). In addition to these more complex phenotypic screens, also screens interrogating biological pathways have been used successfully for drug and target discovery. An example is the porcupine inhibitor LGK974 which targets an acyltransferase in the Wnt signaling pathway and is currently in phase 2 clinical trials for Wnt-dependent cancers (Liu et al. 2013). The inhibitor was found in a screen for inhibitors of Wnt secretion using a coculture system of a Wnt-secreting and a Wnt-reporter gene cell line.

4 Changing Landscape of Academic and Pharmaceutical Research

During the period covered by this chapter, there also have been major environmental changes for drug discovery.

4.1 Laboratory Size

At the beginning of the period, the traditional small laboratory illustrated in Fig. 4 was still prevailing where a master (primary investigator) was working with few apprentices (PhDs, postdocs) in relative isolation conducting experiments by hand and with relatively simple apparatus. Today laboratories are extensively computerized and automated (Fig. 5). With the advent of target-based drug discovery, testing of drugs in whole organism was replaced increasingly by in vitro methods with binding studies probably reaching the bottom of complexity. Today

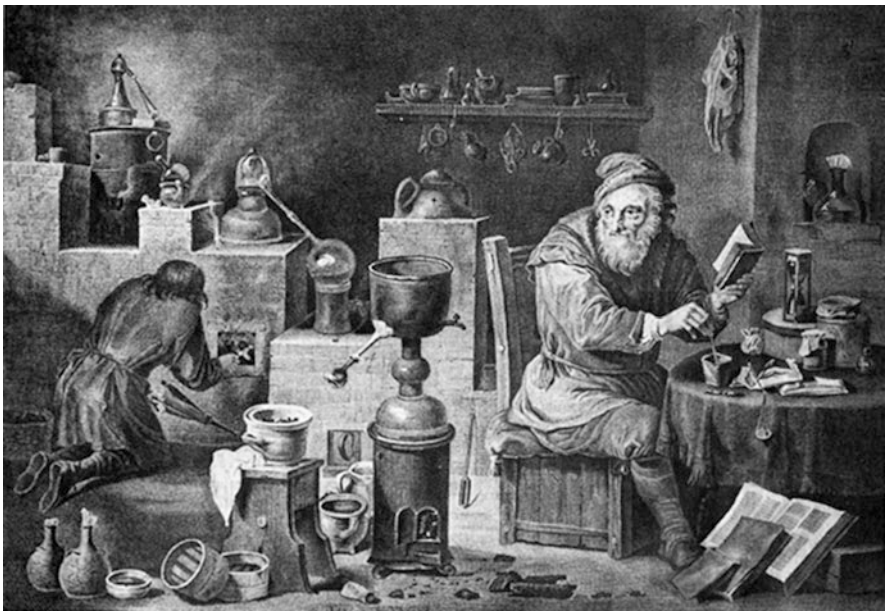

Fig. 4 "Der Alchemist" engraving by Pérée from a painting of David Teniers (1610–1690) illustrating the structure of small labs still retained today

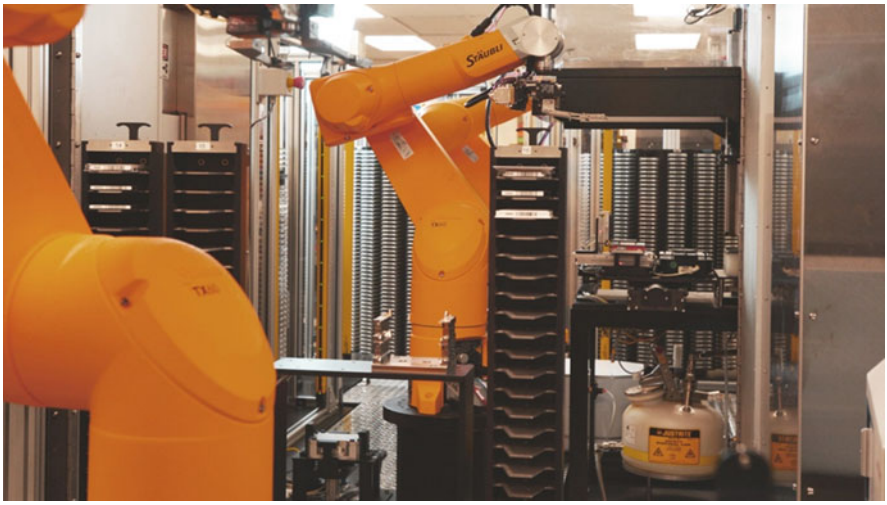

Fig. 5 Fully automated high-content high-throughput screening laboratory, Novartis Institute for Functional Genomics, La Jolla, USA

the complexity of the assay systems has increased again from high-throughput screens on purified proteins to high-content cellular screens allowing evaluation of the effects of new drugs on cells and within them on specific biological pathways.

4.2 Research Center Size and Distribution

In the 1990s it was fashionable to have scientists work in biotech like small groups that could be geographically disseminated under the assumption that their creativity would be better than in large research centers. More recently two factors have caused pharmaceutical research to be increasingly concentrated in large centers (campuses) with thousands of scientists working in walking distance from each other and as close to academic centers of excellence as possible (see below). The first factor is technological: some of the equipment needed to conduct modern drug discovery is very expensive and requires large infrastructures. Examples are robotized screening facilities, compound archives, state-of-the-art animal facilities, high-end microscopy, NMRs, and other analytical tools for chemistry. These tools are deployed in technological platforms that have become too onerous to be multiplied within the same organization at too many geographical sites. On the other hand, for a large pharmaceutical company, it is important to have access to top-quality talent in diverse cultures so that today they usually have Asian, European, and US hubs.

The second factor is biological. As our knowledge of biology grows, it becomes apparent that evolution has been reusing biological components and processes in different environments and in different organs and tissues. This applies prominently to proteins, signaling processes, and cellular pathways. Furthermore, diseases thought to be distinct because of different symptoms and occurrence in different organs turn out to have common molecular pathway malfunctions that simply cause different phenotypes but can be addressed by causal mechanism-specific interventions rather than by trying to correct the symptoms as was mostly the case in the past. An increasingly important consequence of this evolutionary conservatism at the molecular mechanism level is a trend to increasingly classify diseases not by symptoms and organs they occur in but by the causal mechanisms. This changed disease classification method is most advanced in the field of oncology but visible also in immunological disorders. This means that a successful research organization must have experts both in human disease and experts understanding molecular disease processes in all areas they want to be active in. As a drug affecting specifically a disease mechanism might be beneficial in diseases in different organs and with different symptoms, it is essential that scientists of different therapeutic areas interact closely to increase the probability that all potential applications of a drug are found thereby multiplying the clinical use and the returns on the investment. Drawbacks biotech companies must try to solve are that they often cannot afford all technologies needed for drug discovery and, as they

are often focused on one or few indications, that multiple applications of an innovative therapeutic mechanism they discovered will be found and exploited by others.

4.3 In-House and Outsourced Research, Academic Collaborations, and Consortia

Recently a trend has been seen in some large pharmaceutical companies to outsource increasing parts of their research activities under the assumption that they can buy the research products that they need for their commercial success. If the aim is a short-term improvement of the bottom line, then such a systematic outsourcing of research might be successful. If the longer survival of the company is the goal, then the outsourcing strategy might be a bad idea. Biomedical sciences and technologies are highly multidisciplinary scientific activities where the knowledge increases exponentially as was illustrated earlier in this chapter with the evolution of the knowledge about receptors, proteins, and pathways within the last 50 years. The same applies to causes of diseases. In order to understand and make use of the evolving knowledge for significant medical advances, there is only one way: participating in the science with your own scientists. People not participating cannot reach the level of understanding, and even if they tried to reconstruct it from the literature, they would have a significant time disadvantage in addition. Unless a company has a critical mass of scientists participating in all the areas it considers strategic, it will not be able to recognize where to "buy" in a timely and competitive way leading to a continued erosion of their pipeline.

A second essential reason why a critical mass of own scientific research is needed is that many or most of the technological and basic scientific breakthrough needed for pharmaceutical breakthrough relevant for patients occur at academic institutions. As mentioned the only way to recognize and understand the relevant science in a timely fashion the industrial scientists needs to be in close interaction and collaboration with academic scientists so that they will be able to translate the academic breakthroughs into medical breakthroughs which is the only goal of industrial biomedical scientists. Successfully achieving this goal is the only strategy ensuring the long-term success and survival of the company while delivering an essential service to patient and society.

Some scientific questions are too complex to be solved by individual scientists and small laboratories. Examples are systems biology or the quantitative description of cellular-, tissue-, organ-, or entire-organism processes to allow computer simulations that are adequate to predict how these systems will behave. Other examples are the Human Brain Project of the EU or The Cancer Genome Atlas of the USA. These projects are big science projects only possible if large numbers of scientists agree on complementary work programs, data standards, and common database formats. Such topics are sometimes addressed by consortia in public-private partnerships. One directly relevant to drug discovery is the "The Biomarkers Consortium" (Wholley 2014) where scientists from academia, the pharmaceutical

industry, and the US Food and Drug Administration (FDA) work together under the leadership of the Foundation for the NIH to explore and validate relevant biomarkers that could significantly improve the predictability and efficiency of clinical studies.

So in conclusion scientists from academia, the regulatory agencies, and the pharmaceutical industry all contribute to innovative drug discoveries, and, in order to translate the basic science findings into new medical treatments that fulfill unmet patient needs, the pharmaceutical industry needs a critical mass of excellent scientists working in many therapeutic fields, disciplines, and technologies.

4.4 Me-Too Drugs vs. Medical Breakthrough

In the previous section, medical breakthroughs are emphasized. Yet it is mostly easier to make me-too drugs or drugs that are copying an existing one without major additional medical advantage. (A second-generation drug with significant medical advantage is not a me-too but a breakthrough.)

The reason for the imperative to generate breakthrough medical pharmaceutical advances is due to a, to our minds, positive development in our societies. Firstly, pharmaceutical treatments are paid in one way or another by society. Secondly, pharmaceutical treatments work increasingly well, eliminating symptoms, improving the course of diseases, or prolonging life up to achieving complete cure. For these reasons society wants innovative drugs, but there is a limit to how much it can or wants to pay for them. While in the past it was possible to get approval and a market price for me-too drugs, today increasingly society and the payers of medicines are only prepared to pay for significant medical advantages or innovative drugs. Innovation in this context is defined only from one point of view: what needed medical advantage does a new medicine bring to a patient that was not available before. So innovation is not a new molecule, pathway, or target per se *unless* it delivers the medical advance required. A medically relevant innovation can be as "little" as an oral form of a medication in an indication where before only an intravenous injection or constant infusion was available or as "much" as life-prolonging therapies such as imatinib in chronic myelogenous leukemia. In the best case a new medicine is lifesaving as in the case of antibiotics overcoming emerging resistances of infective organisms. Other effective classes of medicines where innovation is urgently needed are the ones that can prevent the occurrence of diseases such as vaccines.

In conclusion, increasingly societies will be unwilling to pay a premium price for undifferentiated even though patented me-too drugs. Research-based pharmaceutical companies with a long-term strategy have a higher probability of success if they build the culture and expertise for biomedical breakthrough innovation addressing the many still unfulfilled medical needs.

4.5 Science Expertise and Culture at the Top

Another important cultural element in the pharmaceutical industry is the expertise of its top management. In the early days, pharmaceutical top management was predominantly composed of chemists, pharmacologists, and medical doctors along with commercial entrepreneurs. But in more recent times, it could be seen that the weight of the scientific disciplines in top management has declined in favor of very predominantly commercial/marketing and financial expertise to the point of being nearly nonexistent in some companies. This is a trend that is dangerous for research-based pharmaceutical companies that envisage a long-term strategy because the key strategic and pipeline decisions must be rooted in scientific, technological, and medical expertise. Both of these are essential to understand patient's needs and how to meet them with medicines that are highly sophisticated packages of scientific information incorporated into biologically active molecules. In view of the immense resources needed to discover and develop new medicines, it is of course important to include commercial expertise in top management but not to the exclusion of scientific expertise.

In conclusion, if a research-based pharmaceutical company wants to be sustainable for the long term, it is essential to have a strong and influential science, medical, and technological component in top management balanced by long-term visionary commercial expertise.

4.6 Productivity

Despite the many great successes of the pharmaceutical industry, there has been an apparent decline in R&D productivity during the past decades manifested by decreasing numbers of drugs approved per billion US dollar spent (Scannell et al. 2012). Paradoxically, a major reason for the apparent decline lies in these past and present achievements of the industry. The ever-growing number of successful drugs inherently increases the scientific, medical, safety, and regulatory hurdles that have to be overcome for new therapies. In addition, with a patent life of 20–25 years and an average preclinical/clinical drug development time of 10 or more years, a proprietary drug can be marketed by a company only for about 10–15 years. A large pharmaceutical company with annual sales of 10–30 billion dollars, therefore, strives to constantly invent a new drug portfolio of the same size (or ideally more) within a 10–15-year time frame just to maintain overall sales figures. To accomplish this, companies typically invest 10–20% of their revenues into R&D activities. And for these activities, the incentive to discover biomedical breakthrough drugs is high as outlined above. Moreover, according to a recent analysis, the first-, second-, and eventually third-in-class drugs will capture more than 90% of the market value in most therapeutic areas (Schulze and Ringel 2013), and thus the focus of many pharmaceutical companies today is to discover and develop first-in-class and best-in-class drugs. There was a widespread trend in the field during the late 1990s and early 2000s to industrialize drug discovery and to

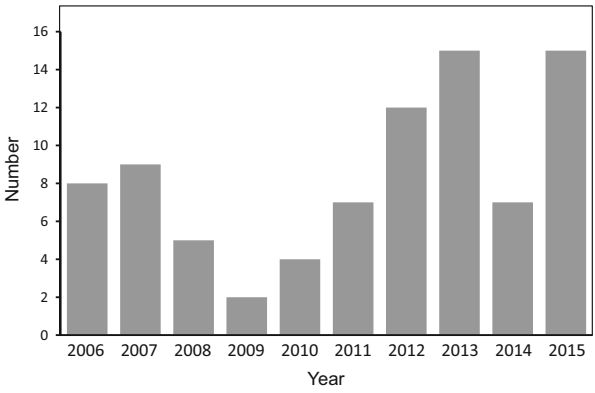

Fig. 6 Number of 1st-in-class drugs approved by the US Food and Drug Administration during the past 10 years

mold it into a linear process comprising a number of separate phases or process steps (target identification, tool production and assay development, hit finding and validation, hit-to-lead, lead optimization, preclinical development). Accepting a priori a highly increased attrition rate in the research phase, the assumption was that brute-force and ever larger numbers of projects and high-throughput experiments would drive productivity. Today, the pharmaceutical industry has largely taken a step back from this brute-force approach realizing that it rather hampered creativity, innovation, and ultimately productivity. Instead, the focus is now much more on science-driven approaches in areas of high unmet medical need where basic science has laid a good foundation for a sufficient mechanistic understanding to allow successful drug discovery. In addition, the industry has started to digest the recent revolutionary advances in technologies and genomics resulting in increased knowledge about complex biological systems and human pathophysiology. This is also reflected by the sharply rising numbers of approved breakthrough therapies by the US Food and Drug Administration (FDA) in 2013 and 2014. In addition, also the numbers of first-in-class drugs approved by the FDA have significantly increased in recent years (Fig. 6). Taking into account the long median time of about 20 years for drug discovery from target identification to regulatory approval (Eder et al. 2014), it appears that only now we are about to see the full impact of modern drug discovery approaches and of the information gained from sequencing the human genome on the productivity of the pharmaceutical industry. This will, no doubt, continue for many decades to come and further change the practice of medicine in significant and probably as yet unimaginable ways.

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Functional Genomics in Pharmaceutical Drug Discovery

Robert Adams, Michael Steckel, and Barbara Nicke

Contents

Abstract

Targeted therapies in personalized medicine require the knowledge about the molecular changes within the patient that cause the disease. With the beginning of the new century, a plethora of new technologies became available to detect these changes and use this information as starting point for drug development. Next-generation genome sequencing and sophisticated genome-wide functional genomics' methods have led to a significant increase in the identification of novel drug target candidates and understanding of the relevance of these genomic and molecular changes for the diseases. As functional genomic tool for target identification, high-throughput gene silencing through RNA interference screening has become the established method. RNAi is discussed with its advantages and challenges in this chapter. Furthermore the potential of CRISPR/Cas9, a gene-editing method that has recently been adapted for use as functional screening tool, will be briefly reviewed.

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Keywords
CRISPR/Cas9 · Functional genomics · High-content assay · High-throughput screening · RNA interference (RNAi) · Short hairpin RNA (shRNA) · Short interfering RNA (siRNA)

Abbreviations

Cas9	CRISPR-associated nuclease
CCLE	Cancer cell line encyclopedia
CRISPR/Cas9	Clustered regularly interspaced short palindromic repeats/Cas9
CRISPRa	CRISPR activation
CRISPRi	CRISPR interference
dsRNA	Double-stranded DNA
DNA	Deoxyribonucleic acid
esiRNA	Endoribonuclease-prepared siRNA
FACS	Fluorescence-activated cell sorting
GoF	Gain of function
HCS	High-content screening
LoF	Loss of function
miRNA	MicroRNA
NGS	Next-generation sequencing
NHEJ	Nonhomologous end join
OTE	Off-target effect
RISC	RNA-induced silencing complex
RNAi	RNA interference
sgRNA	(Small) guide RNA
shRNA	Small hairpin RNA
siRNA	Small interfering RNA
UTR	Untranslated region

1 Introduction

In the target-centric approach of drug discovery, identification of novel targets is the critical first step. This approach is very appealing because of its clear logic whereby the putative drug will interfere with the mechanism by which a target is involved in driving the disease (Patel 2013). The recent tremendous accumulation of "biological big data" such as genomics, transcriptomics, proteomics, and epigenomics data especially for complex diseases like neurological or metabolic disorders or cancer certainly is a very valuable source for potential novel biological discoveries. Fehrmann and colleagues (2015), for instance, have just identified novel genes involved in DNA damage repair and genomic instability by reanalyzing 77,840 publically available expression profiles by using the right

bioinformatics tools. The analysis even led them to predict the biological function of gene.

However, to find exploitable susceptibilities in the disease process needs more than analyzing this empirically generated disease association data. To identify new vulnerabilities or opportunities to directly or indirectly influence cellular responses involved in the disease, it requires the knowledge of gene function: which gene products cause the changes in the biology and how they produce their effects (Diehl et al. 2014). For this, even today's vast reservoir of "big data" is not sufficient as there is still too much unknown about how most gene products work, what their protein interaction network is, and how they are activated – this requires more laboratory bench research to generate data about the roles different gene products play in driving or regulating various biological responses in different contexts. Therefore, for successful target-driven drug development, an understanding of the functional disease relevance of the genomic alterations is crucial. With the discovery of RNA interference (RNAi) in mammalian cells (Elbashir et al. 2001), a powerful technique became available to explore these disease-specific genotypic-phenotypic relations in high-throughput large-scale genome-wide fashion (Mohr et al. 2014).

The development of RNAi technology has used the endogenous microRNA (miRNA) system as a guide. Basically, short RNA sequences which are complementary to the target mRNA are used to charge the endogenous miRNA machinery to destroy the gene transcript (Fennell et al. 2014). By now, there are many different RNAi reagents available, both from commercial or academic resources to target every gene of the entire genome such as chemically synthesized short-interfering (si) RNAs or endonuclease-endoribonuclease-prepared (esi) siRNAs or plasmid-encoded short hairpin (sh) RNAs (Boutros and Ahringer 2008; Grimm 2004; Bernards et al. 2006; Surendranath et al. 2013). Assembled in so-called genome-wide libraries, they are used for assessing the effects of loss of function (LoF) for a given disease-relevant phenotype thereby combining the power of genetic screening with phenotypic readouts (Mohr et al. 2014).

Figure 1 summarizes the workflow of an RNAi-based screening project to identify novel drug targets including assay development and optimization, choice of screening in pooled or arrayed format in the primary screen, screen data analysis, and follow-up verification of the results. In this chapter, the strengths and weaknesses of this RNAi screening technology will be discussed. In addition, a short summary is provided of the next-generation functional screening method based on clustered regularly interspaced short palindromic repeats (CRISPR) genome editing.

2 Types of RNAi Used in Screens

RNA interference is an endogenous process used as a term for various gene regulatory mechanisms. It was observed for the first time in 1990 in transgene petunia when overexpression of the chalcone synthase gene resulted in an

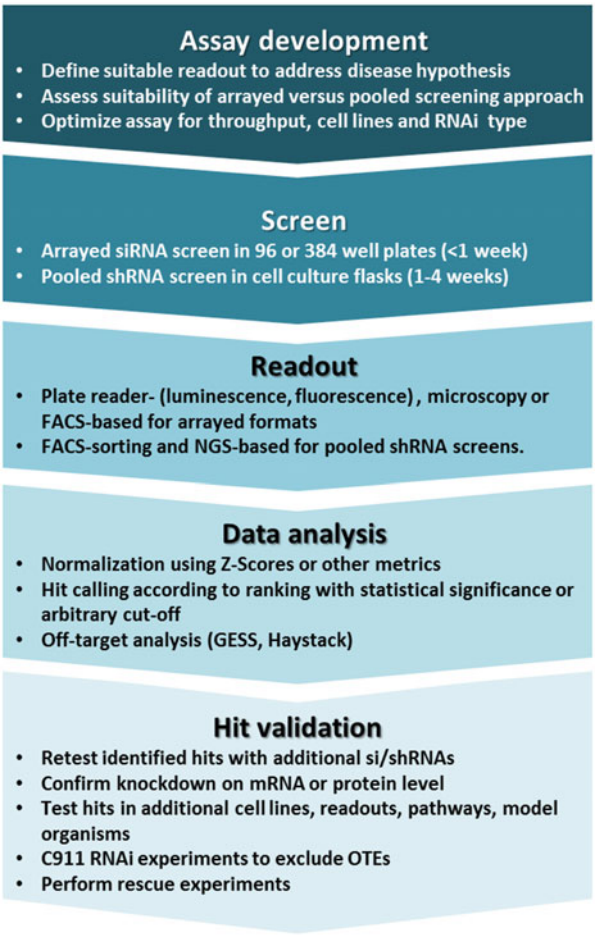

Fig. 1 Workflow for an RNAi screening project to identify novel drug targets

unexpected repression of anthocyanin biosynthesis (Napoli et al. 1990). The effect was termed co-suppression and caused a phenotype of white flowers rather than the expected increase of intensity of the purple blossom color. The mechanism of this unexpected outcome remained unclear until 1998 when Fire and Mello were able to show that the introduction of double-stranded RNA (dsRNA) into the nematode *Caenorhabditis elegans* (*C. elegans*) was critical for the suppression of gene expression (Fire et al. 1998, Fig. 2). This observation was honored with The Nobel Prize in Physiology or Medicine 2006.

Today, there are mainly two types of RNAi routinely used in screening. One involves transfection of synthetic siRNA duplexes that resemble Dicer products (Fennell et al. 2014). siRNAs can only be transfected, as RNA duplexes into dividing cells. For most cell lines, lipid-based formulations work for the delivery of siRNAs. Although siRNA transfection efficiencies are for a lot of cell lines much better than efficacies of transfection of plasmid DNAs, siRNA transfection

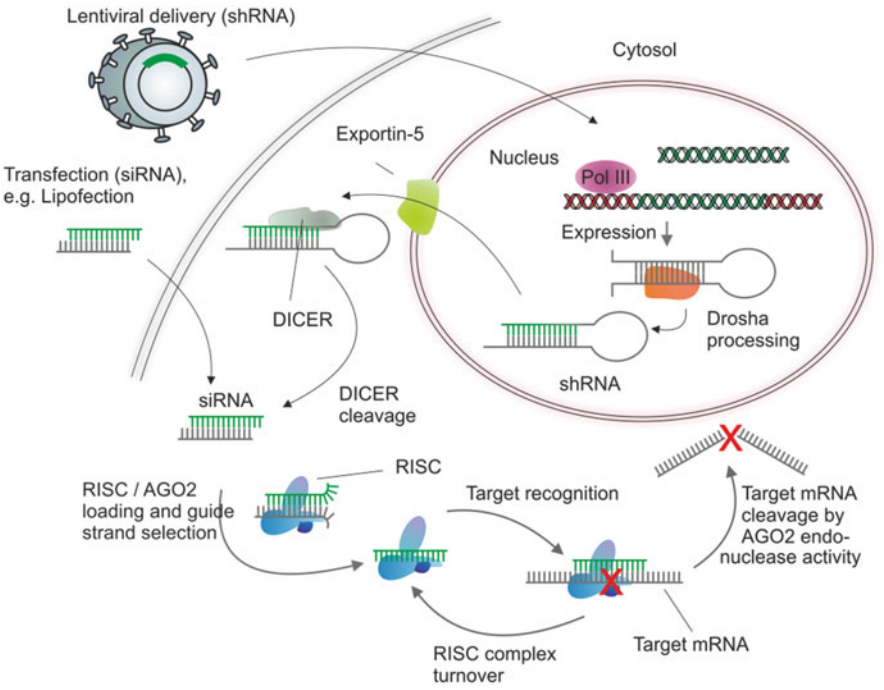

Fig. 2 The RNAi pathway offers different points of interacting with this endogenous regulatory mechanism. The two most common forms of inducing RNAi in cells are the transfection (most often by lipofection, i.e., the delivery of siRNA in lipid vesicles) of naked dsRNAs with 2-nt overhangs at the 3′ ends and the lentiviral delivery of shRNA expression plasmids. shRNAs are processed by Dicer into siRNAs. The guide RNA strand is then incorporated into the RISC (RNA-induced silencing complex), and the complementary strand is degraded. The guide strand then targets RISC to complementary mRNAs, and the Argonaute protein of RISC (Ago2 in mammals) cleaves the target mRNA, rendering it susceptible to degradation by exonucleases (Sigoillot and King 2011)

conditions have to be optimized for each cell line as they can result in toxicity due to high siRNA concentration or transfection reagent toxicity. Another disadvantage of siRNAs is that the siRNA response is limited by stability within the cell and therefore can generally only be used in short-term assays (<96 h). Despite these limitations, synthetic siRNA has been extremely useful in arrayed screening, due to its availability from commercial vendors in whole-genome libraries and its inherent safety as a nontoxic agent (Fennell et al. 2014).

The development of vector-based stem-loop shRNAs and their possible viral-mediated delivery has improved RNAi technology in many areas. shRNAs incorporate into the DNA and thereby exert long-term gene knockdown. The viral transduction allows targeting of hard-to-transfect and nondividing cells, and in vivo studies (Brummelkamp et al. 2002; McManus et al. 2002; Paddison et al. 2002). shRNAs are used in both arrayed and pooled libraries, the latter

being an area that has expanded significantly in recent years. Especially, the high-throughput oligo synthesis and vector cloning technologies have affected quite substantially the further versatile development of shRNA libraries.

3 RNAi Screening Technologies

In order to assess the phenotypic outcome, which results from knocking down a particular gene, various detection methods have been developed. These naturally differ between array-based and pool-based screening formats. For arrayed screening approaches, fluorescence and luminescence plate-reader-based readouts as well as high-content analysis by automated microscopy are routinely used. In contrast to this, pooled RNAi screens are usually assessed by next-generation sequencing (NGS) and/or by and fluorescence-activated cell sorting (FACS) (Fig. 3)

Cell proliferation measurements in an arrayed format can, for example, simply be done by addition of substrates that are readily turned over and metabolized by living cells into fluorescent or luminescent products. Many of these so-called homogenous assays are available probing different parameters within a cell

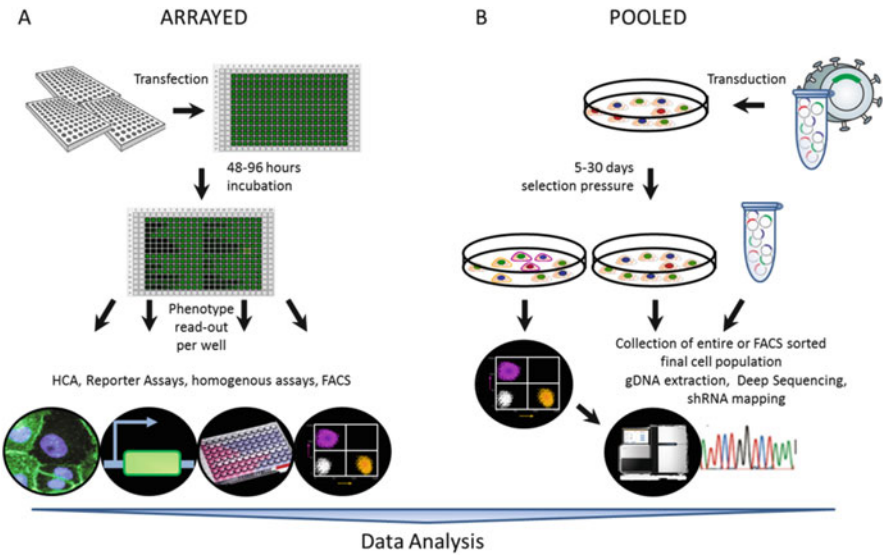

Fig. 3 RNAi high-throughput screening approaches. (**a**) Arrayed screens transfect siRNAs or transduce shRNAs as singles/well. This approach is usually short term (<96 h). Various fluorescent-, luminescent-, or absorbance-based readouts can be determined. (**b**) For pooled screens, shRNAs are pooled as libraries and virally transduced. Forty-eight hours post-transduction, populations are selected for stable integration of the shRNAs for an extended time (5–30 days). Possible assays are FACS sorting (for reporter regulation or expression of readout specific proteins) or viability of surviving cells. In the final cell population, enrichment or depletion of specific shRNAs can be detected by deep sequencing of gDNA and comparison to shRNA representation in initial pool

(i.e., intracellular reductive potential or ATP levels), which in turn can serve as a surrogate of cell number or proliferation. Any of these assays might be compromised if knockdown of a particular gene directly interferes with the parameters addressed in the readout system. One can, for example, imagine that knockdown of a gene involved in ATP metabolism results in a reduced signal even though cell number per se might not have been affected. Therefore, the type of readout has to be carefully adapted to the biological question asked.

The most accurate way to determine cell number and other phenotypic changes upon knockdown of a target is by high-content screening (HCS). Usually cells are labeled with markers, which can be detected by fluorescence microscopy. Theses markers can be, for example, fluorescent dyes that stain DNA or fluorophore-labeled antibodies directed against any given target. Several different markers that fluoresce at different wavelengths can be used at once to address several biological processes simultaneously within the same well by using different detection channels. This information can be complemented by phase contrast or transmission light microscopy to yield complex information on the cellular level. Usually cells that adhere to the bottom of multi-well plates are investigated by HCS, but it is also possible to record several Z-stacks (optical cuts through the Z-dimension) with the aim to capture additional information.

Also several 3D screening approaches using HCS have been reported in the literature. Wenzel et al., for example, developed a 384-well-based screening platform in which they grow multicellular tumor spheroids (one spheroid per well), which allows them to reproduce several parameters of the tumor environment, such as oxygen and nutrient gradients and the development of dormant tumor regions (Wenzel et al. 2014, Fig. 4). In this report a compound library was screened, but it is easily conceivable that a similar assay setup could also be used in an RNAi screen. In order for the shRNAs to penetrate all cells in 3D multicellular structure, such as spheroids, cells are usually first seeded and transduced in a 2D monolayer. Inducible shRNAs are best suited to address the phenotypic effect gene knockdown has in 3D cell culture as one can induce knockdown after spheroid formation is completed. Using constitutive shRNAs, one would rather address the question whose knockdown prevents tumor spheroid formation.

Large-scale arrayed screening approaches require costly automated robotics to handle sufficient throughput of plates and to enable readout especially when HCS is used. In stark contrast, pooled shRNA screenings do not require robotic setup to perform the actual screening and can be done in any standard laboratory in which infectious viruses can be handled. In a pooled approach, cells are simply infected by a library of shRNAs in large cell culture flasks or dishes. Another advantage of pooled formats is that cells can undergo several doublings as they are not restricted by the limited space in 96- or 384-well plate cavities before the experiment is read out. This is of importance when the phenotype upon gene knockdown needs some time to develop. In general pooled screens are read out by monitoring the amount of shRNA integrated in the genomic DNA of cells at the start of the experiment and at the end of the experiment. This is usually done by next-generation sequencing of

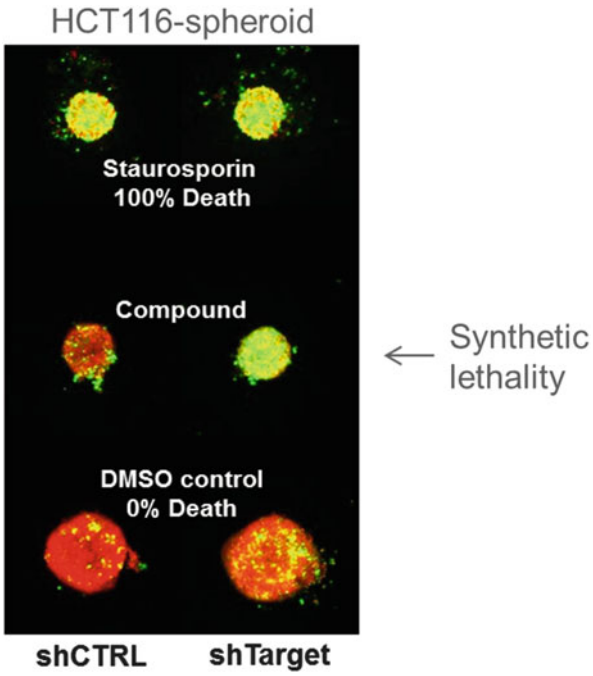

Fig. 4 3D tumor spheroids represent a cell-based model that mimic the situation in a real tumor more appropriately. *Green color* indicates dead cells (Sytox Green assay counterstained by Hoechst). Spheroids targeted by shRNAs only (cells at the bottom) do not show an increased cell death (negative control), while the combination of a specific compound and the targeting shRNA causes death of the cells inside the spheroid (synthetic lethality). Spheroids treated with very high concentrations of staurosporine (multikinase inhibitor that blocks ATP binding) do not survive independent of additional shRNA treatment (positive control, cells at the top). shCTRL is a nontargeting (so-called scrambled) control (figure generously provided by Patrick Steigemann and Gerrit Erdmann, Bayer Pharma AG)

cellular DNA and detection of either the unique shRNA sequence or a barcode, which can also be introduced into the DNA as part of the shRNA construct. As an alternative, also microarray technique can be used to measure abundance of each shRNA. If the amount of shRNA at the start of the experiment is higher than at the end, then this indicates that those cells who had the shRNA introduced into their genome either died or at least grew slower than the control (i.e., an shRNA that does not target a gene/nontargeting vector). This approach is named dropout screening accordingly.

An alternative or additional way to read out pooled screens is by FACS sorting. Cells that, for example, can be engineered to have a reporter gene construct integrated that translates into a fluorescent protein, which then can be sorted for. Subsequent NGS analysis allows for the identification of target genes whose knockdown contributed to activation of the reporter gene.

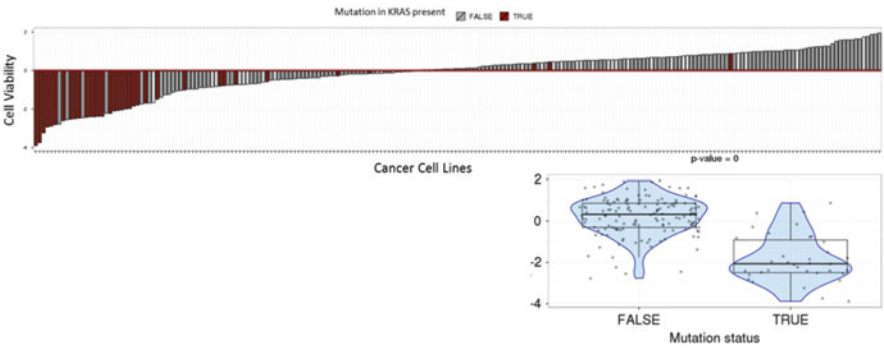

Fig. 5 Example for the correlation of functional cell viability data with oncogenomic data. A KRAS mutation sensitizes a cell line toward the knockdown of KRAS, a phenomenon that is well studied and known as an oncogenic addiction. The viability data is derived from massive parallel shRNA screening in several hundred cancer cell lines (Cheung et al. 2011). The mutation data is provided by the CCLE (Cancer Cell Line Encyclopedia) (Barretina et al. 2012)

3.1 Synthetic Lethality

In recent years the concept of synthetic lethality has been investigated with RNAi approaches. Two genes are said to be synthetic lethal if perturbation (i.e., inhibition) of either one gene alone does not have a phenotypic effect, but perturbation of both genes at the same time results in lethality. This concept can be applied to address several biological questions relevant in drug discovery including the identification of genes whose knockdown is lethal to cells harboring an oncogenic mutation, which itself might not be druggable. Similarly, identification of synthetic lethal partners to cells harboring deleted or inactivated tumor suppressor genes allows extending the target space for oncology enormously.

The Project Achilles at the BROAD Institute applies shRNA dropout screening on a genome-wide scale in several hundred characterized cancer cell lines with the aim of systematically identifying and cataloging cancer cell vulnerabilities (http://www.broadinstitute.org/achilles). Integrating this data with other annotations including the mutational status of cell lines, which is available through the CCLE allows the identification of specific cancer genetic dependencies (http://www.broadinstitute.org/ccle). Figure 5 shows as an example the sensitivity of numerous cancer cell lines toward shRNA-mediated knockdown of KRAS. As expected, the phenomenon termed oncogene addiction (Weinstein 2002) can nicely be demonstrated as cell lines bearing an activated mutation in the KRAS gene are more sensitive toward knockdown of KRAS as cell lines with wild-type KRAS. Using the data generated in Project Achilles, Wilson et al. (2014) showed a synthetic lethal interaction of SMARCA2 expression and SMARCA4 mutation, and Helming et al. (2014) identified ARID1B as the Achilles heel of ARID1A-mutated cancer cell lines.

3.2 Assay Performance, Hit Identification, and Statistics in Plate-Based Screening

In order to assess the quality of an arrayed screening campaign and to identify hits, several statistical measures can be used. First, data has to be normalized to remove systematic errors and to allow comparison of data points that were generated on different plates. Many normalization methods have been used in RNAi screening including Z-Scores and B-Scores. The Z-Score normalization provides information on the strength of each siRNA relative to the rest of the sample distribution and is defined as the number of standard deviations an observation is away from the mean of the plate (or screen). More robust methods, which account better for outliers and systematic plate effects, include the robust Z-Score (number of median absolute deviations) and B-Score, respectively (Brideau et al. 2003). Birmingham et al. (2009) comprehensively summarized and compared various suitable statistical methods to be used for high-throughput RNAi screens. The cellHTS web application (Pelz et al. 2010) is a tool that guides the user through the various normalization steps and facilitates the analysis of high-throughput data sets and is accessible at http://web-cellHTS2.dkfz.de. Hits of an RNAi screen are usually identified by comparison to a negative control or to all samples on the plate (assuming the genes to be targeted in the library are randomly distributed across the plates, i.e., in unbiased screens targeting the druggable (~10,000 genes) or the whole genome). The strictly standardized mean difference (SSMD) is another measure of effect size, which can be used to select hits in an HTS. It is the ratio of the mean to the standard deviation of the difference between two random values each from one of the two groups. For reading out pooled shRNA screens, similar methods as described above can be used. Moreover the pooled approach allows for assessing shRNA dropout or enrichment at multiple timepoints and in several cell lines in parallel.

4 Addressing Off-Target Effects in RNAi Screens

Unintended effects, both false-positive and false-negative screening results, are often intrinsic to high-throughput approaches. They have to be taken into consideration while interpreting the outcome of such screens (Mohr et al. 2014). Unlike genetic approaches that act on the DNA, RNAi decreases mRNA levels or interferes with the translation of the mRNA. Thereby, RNAi-based experiments can suffer from a lack of sensitivity due to incomplete suppression of gene expression or a lack of specificity due to suppression of unintended genes (Sigoillot and King 2011).

Off-target effects (OTEs) can arise from a range of mechanisms comprising both sequence-independent and sequence-dependent effects. Effects that are independently of the siRNA sequence might even be considered avoidable by careful assay development. For instance, can expression of exogenous shRNA disrupt the endogenous processing of miRNA or compete with endogenous miRNA entering the RISC, which causes alteration of the normal gene expression pattern (Sigoillot and King 2011; Khan et al. 2009)? Other unspecific effects can of course be induced by toxic transfection conditions or other stress-causing treatments. Another source for

unintended effects when applying RNAi in mammalian cells is the antiviral immune response leading to the induction of interferons and inflammatory cytokines. Using 21 nt short siRNA sequences instead of longer double-stranded sequences (e.g., used for RNAi in nematodes) has proven to avoid the interferon response (Sigoillot and King 2011). Therefore, carefully testing all the conditions of an RNAi screening experiment, using the lowest possible concentrations of reagents, and including appropriate controls are crucial to avoid these potential unspecific sequence-independent effects during the screen.

However, the more pronounced and less controllable unintended effects originate from sequence-dependent off-target gene regulation. This evolves from partial sequence homology between siRNAs and regions of 3′ UTR of unintended target transcripts and is termed siRNA seed effect. The off-target mechanism by siRNAs is similar to miRNA-mediated gene regulation that that induces target recognition and subsequent translational inhibition through short regions of matches between heptamer sequences (7 nts) of its 5′ ends and regions of the 3′ UTR of multiple target transcripts (Mohr et al. 2014). siRNA-induced OTEs are caused by perfect matches of the so-called seed sequence of the siRNA within the 3′ UTR of the unintended mRNA. The seed sequence is defined as the region of nucleotides 12–18 on the guide strand of the siRNA duplex. A perfect seed match induces a miRNA-like OTE due to triggering a loaded RISC to 3′ UTR recognition. Increased stringency is gained through the probability of a gene to be off-targeted by the presence of multiple seed-matches within the 3′ UTR of a transcript (Birmingham et al. 2006; Ameres et al. 2007).

Although the sequence-dependent OTEs have already been recognized almost as soon as on-target siRNA effects had been discovered (Jackson et al. 2003), acknowledging and finding ways to address them accordingly took some time. Initially, approaches were limited to changing siRNA design features, and chemically modifying the passenger strand was intended to increase the probability of loading only the guide strands into RISC. As with sequence-independent OTEs, experimental conditions should also be optimized to employ the lowest effective dose of siRNA (Birmingham et al. 2006; Martin and Caplen 2007).

More recent approaches concentrate on developing novel bioinformatics tools and additional experimental approaches to address sequence-dependent OTEs. Novel analysis algorithms such Genome-wide Enrichment of Seed Sequence matches (GESS, Sigoillot et al. 2012) and Haystack (Buehler et al. 2012a, b) use different strategies to identify unintended targets based on siRNA seed sequence-3′ UTR relations.

As an experimental approach to address these OTEs, a method of mismatched siRNA design has been proposed, termed C911. For each siRNA, a control siRNA can be designed in which bases 9–11 are substituted for their complement bases, but the seed sequence is kept identical (Buehler et al. 2012a, b). Seed-based false-positive siRNAs maintain the phenotype when C911 is changed, while true-positive hits do not (Fig. 6).

An established experimental strategy for the validation of RNAi screen results is using several different RNAi reagents targeting each gene, assuming that different

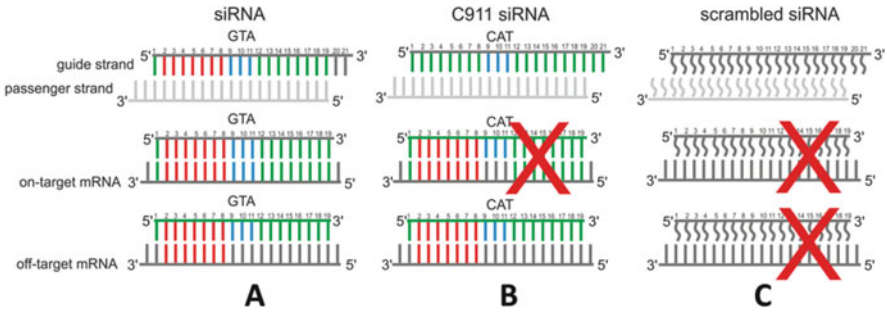

Fig. 6 On- and off-target effects of siRNAs and their controls. An siRNA (**a**, *left panel*) consisting of two complementary 19-mers of RNA (with two-base overhangs) is divided here conceptually into the 5′ end of the antisense strand (*red*), the middle of the siRNA (*blue*), and the 3′ end of the antisense strand (*green*). An on-target siRNA is reverse complement of the target mRNA sequence intended to be downregulated (**a**, *middle panel*), but this siRNA can also match to the seed sequence of a 3′ UTR of other mRNAs and can downregulate as in their off-target (**a**, *bottom*). Because it lost full complementarity in the middle three bases (*blue*), the C911 mismatch control (**b**) does downregulate the targeted mRNA, but because the antisense and sense strand seed sequence is still intact, it maintains the off-target effects of the original siRNA. The comparison of effects elicited by the original siRNA and the C911 mismatch control allows to distinguish phenotypes that are due to downregulation of the intended target rather than off-target effects. A scrambled siRNA (**c**) eliminates the match to the target mRNA and thus will not downregulate it but also eliminates the off-target effects due to matches to the seed sequence (however, it often creates new off-target effects against the new seed sequence) (Buehler et al. 2012a, b)

reagents will contain different seed sequences. For siRNA screens, seven or more independent reagents per gene might be assayed; for pooled shRNA screens, some investigators screen libraries using more than 15 constructs per gene (Bassik et al. 2009).

Taking these possible pitfalls of RNAi screening in consideration when designing and analyzing the screening project and planning follow-up strategies will be absolutely crucial for the success of an RNAi-based screen for novel targets. Screens against pathways or cellular processes that involve large numbers of different components (cell proliferation, cell death, cell signaling) may be especially prone to a high rate of false-positive results (Sigoillot and King 2011). Therefore, choosing more complex readouts or performing complementary screens might reduce the false-positive hit rate. The emphasis however lies on the thorough verification of screening results.

5 CRISPR/Cas9 Target Screening

A new technique has been developed as an alternative approach for RNAi-based LoF genetic screens. Identified as a prokaryotic viral defense response that utilizes clustered regularly interspaced short palindromic repeats (CRISPR) to target DNA, CRISPR/Cas9 has been adapted to be used to create “gene knockouts” by disrupting

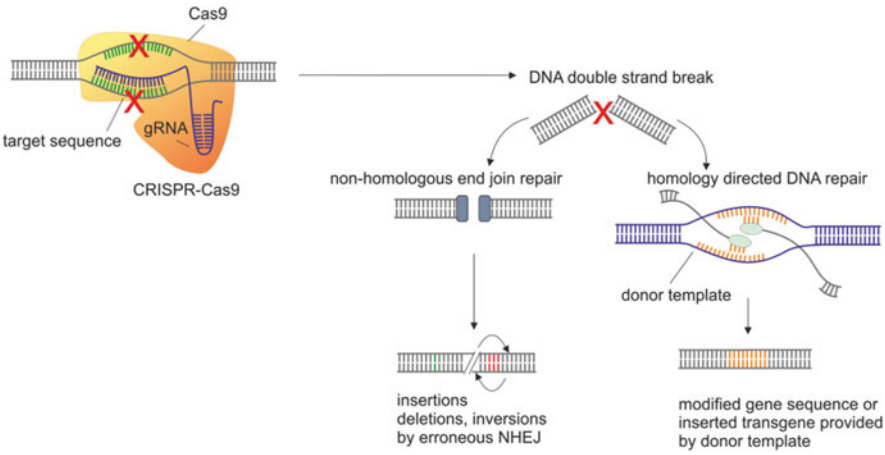

Fig. 7 CRISPR/Cas9 as genome editing tool for LoF approaches. CRISPR/Cas9 introduces sequence-specific double-strand breaks in genomic DNA using different approaches for target sequence recognition. Utilizing error-prone endogenous DNA damage repair mechanisms (NHEJ: nonhomologous end joining), insertions and deletions, which cause frameshifts, are introduced. This subsequently leads to complete knockout of a targeted gene. A second approach uses the homologous recombination pathway. Thereby, a plasmid is used that contains sequences homologous to the double-strand break flanking regions and additionally any desired sequence that is subsequently integrated into the gene. This enables not only silencing of genes by introducing frameshifts but also the introduction of base-specific substitutions enabling reversion of mutations, introduction of mutations, alteration of promoter sequences (thus activation and inactivation of gene expression), and other applications

genomic DNA permanently (Sander and Joung 2014). It has been found that incorporating a sequence of just 19 nucleotides called single-guide RNAs (sgRNA) into a short RNA strand of about 70 bases will target the CRISPR associated bacterial nuclease Cas9 to the specific region of genomic DNA. CAS9 induces then there DNA double-strand breaks which is followed by error-prone nonhomologous end joining (NHEJ) repair. This leads to small insertions or deletions, most of which generate frameshift mutations often causing the knockout of the gene of interest (Jinek et al. 2013; Mali et al. 2013, Fig. 7). Thus, in analogy to RNAi, applying specific design rules, sgRNAs can be designed to target any gene of the genome (Doench et al. 2014; Sanjana et al. 2014). However, in contrast to RNAi, this targeting knocks the genes out completely, thus addressing one of the limitations of shRNA screening – the problem of lacking sensitivity due to incomplete suppression of gene expression (Fennell et al. 2014). A few labs have utilized CRISPR in LoF screens already successfully in a manner similar to shRNA pooled screening using sgRNA viral expression constructs (Wang et al. 2014; Shalem et al. 2014; Zhou et al. 2014) showing that the infrastructure established for shRNA-based screening can be easily adapted for these genome-wide knockout screens (Moore 2015).

CRISPR is actually applicable more broadly than RNAi since it can be used to disrupt nonprotein coding regions of the genome. Also, some labs have modified the Cas9 protein to eliminate the nuclease activity and introduce other functions so that CRISP methods can also provide to shut off or enhance transcription and provide a platform for gain-of-function screens (GoF) (Cheng et al. 2013). Gilbert and Horlbeck et al. reported 2014 the development of two related tools called CRISPRi and CRISPRa to repress or induce, respectively, individual transcripts with minimal off-target effects, which provide complementary biological insights through both loss- and gain-of-function genetic screens (Gilbert Luke et al. 2014).

There are of course also for CRISPR/Cas9-based gene-editing potential off-targeting effects to be anticipated. Because of the experience with RNAi-based screening, researchers try to address these with highest priority (Doench et al. 2014; Sanjana et al. 2014; Hendel et al. 2015). One factor is the exposure of the genome to the nuclease. Although cells transfected with the Cas9 nuclease without co-expressing single sgRNAs have not been found to have an increased mutagenesis rate at any of the interrogated sites (Hendel et al. 2015), studies have found that changing exposure time to the nuclease in the presence of sgRNAs can improve the "specificity ratio," defined as the ratio of on-target activity to off-target activity (Hendel et al. 2015). Also a fusion of a catalytically inactive Cas9 to the FokI nuclease (Cas9-FosI or fCas9) has been found to improve the specificity of the nuclease tremendously (Guilinger et al. 2014). Because the sequence of a genome is not random, another potential off-target causing factor is if there are homologous sites to the intended target site found in the genome and how many (Hendel et al. 2015). This has been one reason to further improve the design of the already existing sgRNA libraries (Doench et al. 2014; Sanjana et al. 2014; Hendel et al. 2015)

As for every novel technology, there is still a lot to be learned and tested, but clearly CRISPR provides already a core technology on which to build new exciting tools to explore the function not only by interrupting protein coding genes but also by specifically modifying them.

6 Conclusion

In conclusion, RNAi and recently also CRISPR-based high-throughput functional screening has clearly had an impact on our biological understanding of diseases as well as for the discovery of targets most notably perhaps for cancer treatment. With the growing knowledge about how to address the intrinsic flaws of the RNAi screening technologies, the confidence in the validity of RNAi screening results and the enthusiasm about the technology will increase again (Fennell et al. 2014). Specifically, investing in thorough assay development including the choice of reagent and assay design, subsequent sophisticated data analysis and data integration, and very careful follow-up experimental validation, large-scale RNAi screens will be successful at uncovering new genes, signaling pathways, and gene networks involved in disease mechanisms and will continue to be a valuable experimental

tool (Mohr et al. 2014). Developing novel functional genomic techniques such as CRISPR/Cas9 alongside and evaluating and addressing potential pitfalls early on will provide powerful complementary tools for successful identification of novel drug targets in an unbiased way.

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Emerging Target Families: Intractable Targets

Stefan Knapp

Contents

Abstract

The druggability of a target is defined by the likelihood of a certain target binding site to be amendable to functional modulation by a small molecule in vivo. Thus, druggability depends on the ability of the developed small molecule to reach the target site, the properties of the ligand binding pocket and our ability to develop chemical matter that efficiently interact with the drug binding site of interest. Historically enzymes have been the main drug targets because the inhibition of their activity can be easily assayed and catalytic centres are often attractive drug binding sites. However, despite considerable effort, a number of classical enzyme families have not been successfully targeted. More recently protein–protein interactions received considerable attention and several clinical inhibitors have now been developed. Despite the considerable progress made expanding target space, a large number of targets with a very strong

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rationale for targeting remain intractable. In the following chapter I will summarize progress made in developing inhibitors for challenging drug binding sites and emerging target families.

Keywords
Druggability · Epigenetic reader domains · Protein interaction inhibitors · Phosphatases · RAS · Structure based design

1 The Concept of Druggability and Properties of Drug-Like Molecules

Lipinski et al. noted in 1997 a number of shared physicochemical properties of drug candidates successfully entering phase II clinical trials (Lipinski et al. 2001). These parameters defined in this study are now known as the 'rule of 5' (Ro5). Based on the initially defined Ro5 criteria, a pharmacological small molecule is more likely to be active when administered orally if it has no more than one violation of the following criteria: It should not have a) more than 5H-bond donors, b) a molecular weight larger than 500, c) a cLogP (calculated octanol/water partition coefficient) larger than 5 and d) no more than 10 hydrogen bond acceptors. In a subsequent study, the molecular weight constraint has been questioned, and this parameter is nowadays often replaced with the related polar surface area criteria, which should ideally less than 140 $Å^2$.

In addition, the number of rotatable bonds (less than 10) has been identified as an important predictor for compounds that are orally active (Veber et al. 2002). During lead development the molecular weight and lipophilicity are usually increased by medicinal chemists in order to increase potency and target specificity. As a consequence, stricter requirements are usually applied to lead compounds (rule of three, Ro3) which are defined by an octanol/water partition coefficient log *P* not greater than 3, by a molecular mass less than 300 Da and by the presence of not more than three hydrogen bond donors and not more than three hydrogen bond acceptors as well as not more than three rotatable bonds (Congreve et al. 2003). There are however many exceptions to these rules, in particular for drugs that act at protein interfaces such as taxanes that target tubulin or rapamycin, targeting the interaction of the kinase mTOR/FRAP with FKPB12 (FK506 binding protein). These very successful approved drugs largely exceed the molecular weight/polar surface area constraint as well as the recommended number of hydrogen bond donor/acceptors that would be expected to result in a bioactive drug. However, both rapamycin and paclitaxel are natural products and may have been adapted through a natural selection process to function in vivo despite their poor drug-like properties (Fig. 1).

The physicochemical constraints of drug-like molecules naturally constrain the type of binding sites that can be targeted. Thus, large and shallow surfaces are not likely to interact with drug-like small molecules with sufficient potency and highly polar surfaces will result in interacting ligands with poor cellular activity. Several prediction tools have been developed to assess the druggability (likelihood of

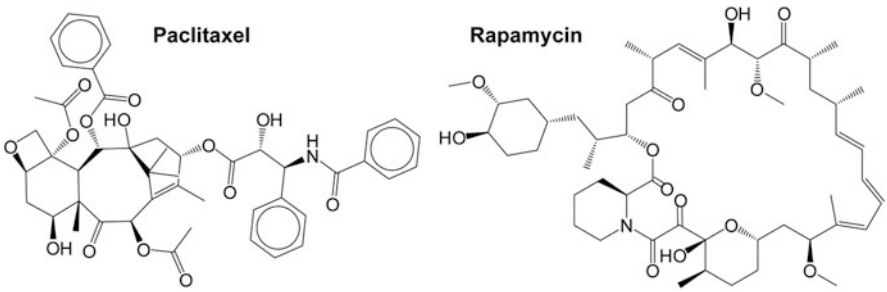

Fig. 1 Examples of approved bioactive 'non-Lipinski' drugs targeting protein interfaces

developing compounds with appropriate in vivo bioavailability, efficacy and safety profiles) or chemical tractability (likelihood developing a potent in vitro inhibitor) of target binding sites (Halgren 2009; Keller et al. 2006). The developed tools include methodologies estimating the 'maximum achievable affinity' (Cheng et al. 2007) or are based on experimental techniques that correlated with pocket properties, for instance, NMR screening hit rates (Hajduk et al. 2005).

A remarkable recent study examined potential drug binding pocket in the protein databank (PDB (http://www.rcsb.org/pdb/home/home.do)). The study identified 290,000 suitable binding pockets present in 42,000 crystal structures that were available during the time of analysis (Sheridan et al. 2010), suggesting that there is a large space of potential drug binding sites that has not been explored. Indeed, the currently accessed target space was recently compiled surveying 27,000 documents including patents. This study identified assay data for 1,736 human proteins that were targeted by 823,179 unique chemical structures (Southan et al. 2011). However, the top 278 most actively pursued targets were classical enzyme or membrane protein drug targets covering 90% of the identified compounds, suggesting that protein interaction inhibitors and nonclassical targets represent still a very small niche area. This is particularly evident analysing the current targets of approved drugs. In 2002 an analysis by Hopkins revealed 399 nonredundant protein targets. However, targets of drugs that are actually marketed constituted only 120 proteins (Hopkins and Groom 2002). Nearly half of the targets did fall into six different target families: G-protein-coupled receptors (GPCRs), serine/threonine and tyrosine protein kinases, zinc metallopeptidases, serine proteases, nuclear hormone receptors and phosphodiesterases. The rate of new target discovery is still slow (Overington et al. 2006). During the past decade, 19 new chemical entities and biologics have been approved on average each year. From this set, only four new drugs are developed against previously unexploited molecular targets (Rask-Andersen et al. 2011). In the following, I have selected a number of challenging target families with strong biological rationale for drug development.

2 Example 1: Phosphatases, Classical Enzyme Targets with Low Druggability

Protein tyrosine phosphatases (PTPs) counterbalance the action of protein kinases and are essential enzymes regulating cell signalling. Similar to protein kinases, many phosphatases are deregulated in disease making a compelling case target these enzymes. There are 107 PTPs encoded in the human genome that can be grouped into four major families: classes I, II and III of cysteine-based PTPs and aspartate-based PTPs. The class I cysteine-based PTP group contains the phosphotyrosine-specific enzymes that are usually referred to as 'classical PTPs' (Alonso et al. 2004). Dual-specificity PTPs and low molecular weight PTPs have very shallow and charged binding sites making inhibitor development a challenging task. Within the classical PTP family, binding cavity properties are more favourable but cellular activity and selectivity remain major challenges (Barr 2010).

Two targets received most of the attention developing PTP inhibitors to date: PTP1B and SHP2 (PTPN11). The phenotype of the PTP1B knockout mice sparked drug development interest as these animals were healthy but displayed enhanced sensitivity to insulin and resistance to a high-fat diet-induced obesity, suggesting that selective PTP1B inhibitors could be beneficial treating both type II diabetes and obesity (Elchebly et al. 1999). In addition, possible applications of PTP1B inhibitors in oncology have been suggested by transgenic mouse studies that showed that PTP1B functions as a positive mediator of the ErbB2 tyrosine kinase signalling leading to breast cancer development and tumour metastasis (Julien et al. 2007). However, PTP1B is highly similar to TC-PTP which share 72% sequence identity and 94% identity considering active site residues. In contrast to PTP1B, TC-PTP knockout mice die shortly after birth as a result of anaemia, hypersensitivity and widespread inflammation strongly suggesting that inhibiting this PTP should be avoided (You-Ten et al. 1997).

There has been also significant drug discovery interest in the development of SHP2 inhibitors for the treatment of cancer. Similar to cytoplasmic tyrosine kinases, SHP2 is kept in an inactive state by its N-terminal SH2 domains (Hof et al. 1998). Binding to phosphorylated tyrosines released the SH2 domain block on SHP2 activity, activating this phosphatase. Mutation in SHP2 (PTPN11) has been associated with Noonan and LEOPARD syndrome and development of several cancer types, and most significantly activating SHP2 mutations are found in 35% of patients with juvenile myeloid leukaemia (JML) (Chan et al. 2008). In addition, high SHP2 expression levels have been associated with increased leukaemia and breast cancer risk (Xu et al. 2005; Zhou et al. 2008). Similar to PTP1B, also SHP2 is closely related to an anti-target: SHP1, a highly similar PTP which is mainly expressed in the haematopoietic system. SHP1 loss of function has been associated with severe autoimmune and immunodeficiency syndrome (Shultz et al. 1997).

The structure of PTP1B with a dually phosphorylated peptide derived from the insulin receptor identified a secondary phosphotyrosine binding pocket (Salmeen et al. 2000) and a potential binding site that can be targeted by small molecules. This secondary pocket is present in a number of PTPs and may have different

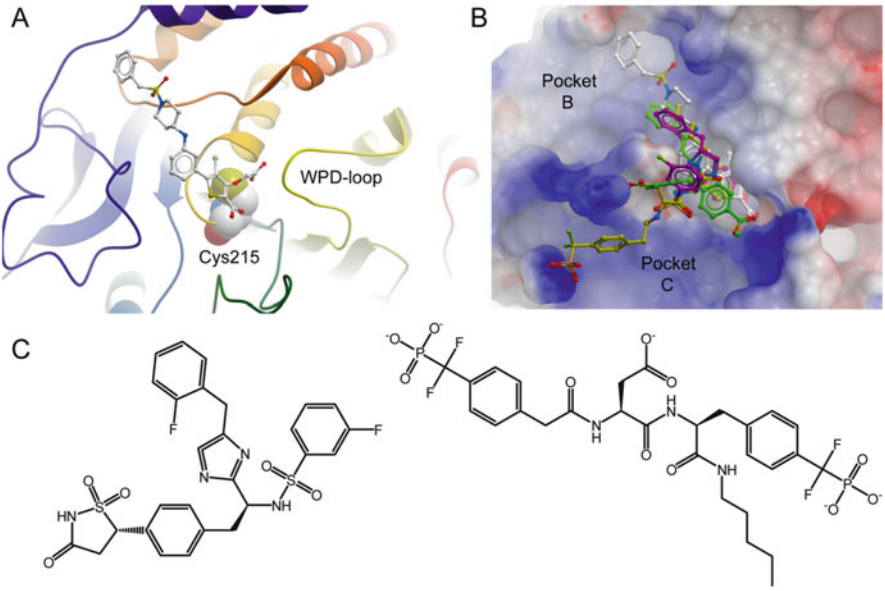

Fig. 2 Binding modes of PTP1B inhibitors. (**a**) Main structural elements of the PTP catalytic domain. The active site cysteine residue (Cys215) is highlighted in cpk representation. An inhibitor targeting the secondary phosphotyrosine binding site (pdb-ID: 2qbp) is shown in *ball* and *stick representation*, (**b**) overlay of a number of PTP1B inhibitors (pdb-IDs: 1pxh, 2vew, 2qbp, 2fjn) targeting also pocket adjacent to the primary phosphotyrosine binding site as outlined in Barr (2010) and (**c**) chemical structures of two of the co-crystallized inhibitors

physiochemical properties (Barr et al. 2009). A number of inhibitors targeting this secondary binding site and other binding pockets adjacent to the primary phosphotyrosine binding site have been developed (Fig. 2). One of the most potent inhibitors that makes use of a nonhydrolyzable phosphonodifluoromethyl phenyl group (F2pmp group) has a reported Ki value of 2.4 nM, tenfold selectivity over TC-PTP and excellent selectivity of other members of the PTP family (Shen et al. 2001).

The charged surface of the PTP active sites spurred the search of alternative targeting sites, and a number of allosteric inhibitor development strategies have been reported. Wiesmann et al. reported an allosteric inhibitor that prevents the closure of the WPD loop (Fig. 3). The reported inhibitor targeting this binding site had however only modest activity ($IC_{50} = 8$ μM) and selectivity for TC-PTP (Wiesmann et al. 2004), but it was active in cell-based assays monitoring insulin receptor phosphorylation. In addition, trodusquemine (MSI-1436), a spermine metabolite of cholesterol that was originally isolated from the dogfish shark liver, has been shown to non-competitively inhibit PTP1B but not TC-PTP. MSI-1436 has an IC_{50} value of about 1 μM (Fig. 3). The compound is active in vivo producing a dose-dependent weight reduction and has insulin-sensitizing properties (Lantz et al. 2010). Recently further characterization of this interesting compound revealed that

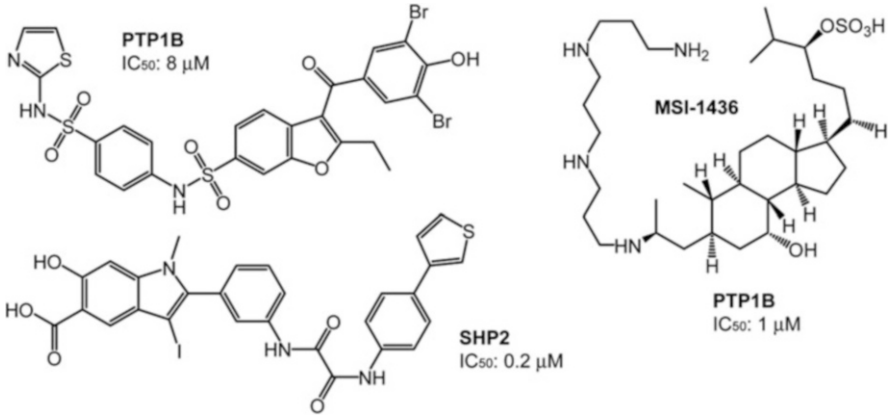

Fig. 3 Chemical structure of allosteric PTP1B inhibitors as well as the potent SHP2 inhibitor 11a-1 (Zeng et al. 2014)

MSI-1436 targets the C-terminal, non-catalytic domain of the long isoform of PTP1B. The PTP1B C-terminus is an intrinsically disordered region of the protein that allosterically regulates PTP1B catalytic activity by interaction with the catalytic domains. In breast cancer, MSI-1436 antagonized HER2 signalling and inhibited tumorigenesis and metastasis in xenografts (Krishnan et al. 2014).

Only few inhibitors have been reported for SHP2 so far. One of the most potent compounds targeting this PTP is NSC-87877 that is cell active and inhibits SHP1 and SHP2 with an IC50 value of 300 nM. Recently, a novel hydroxyindole carboxylic acid-based SHP2 inhibitor 11a-1, with an IC_{50} value of 200 nM and greater than fivefold selectivity against other PTPs, has been reported (Fig. 3) (Zeng et al. 2014).

3 Example 2: GTPases of the RAS Family

The small GTPases of the RAS family (*HRAS*, *NRAS* and *KRAS*) were the first mutated genes discovered in cancer and constitute today the most frequently mutated oncogenes. The high rate of RAS pathway activating mutations that have been detected in the most lethal cancer types has triggered a considerable research interest developing small molecules that interfere with RAS function. However, despite more than 3 decades of intensive efforts, no RAS inhibitor that targets this GTPase directly has reach clinical testing, suggesting poor druggability of these targets (Karnoub and Weinberg 2008; Cox et al. 2014).

GTPases are activated by GTP and inactivated by binding of GDP. This process is tightly controlled by a number of regulatory proteins such as the GTPase-activating proteins (GAPs) as well as GTP exchange factors (GEFs). Mutationally activated RAS usually show impaired GAP stimulation and the mutations stabilize

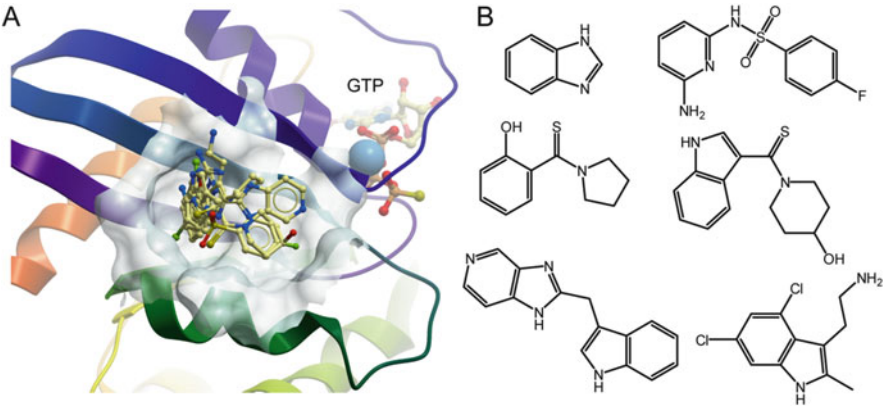

Fig. 4 Fragment binding site identified in RAS. Shown is a superimposition of several fragments co-crystallized with RAS (**a**) as well as the chemical structures of the co-crystallized compounds (**b**). The GTP/Mg binding site is indicated as well

the persistently GTP-bound active RAS state (Bos et al. 2007). The extremely high affinity of GTPases for GTP precluded unfortunately the development of GTP competitive ligands. Other targeting strategies have been therefore attempted. Among the most advanced strategies targeting RAS regulators has been the development of farnesyltransferase inhibitors. This enzyme transfers a C15 farnesyl isoprenoid lipid to its carboxy-terminal CAAX motif in RAS resulting in targeting of RAS to the plasma membrane. Several highly potent inhibitors have been developed that showed remarkable efficacy in mouse models, and several inhibitors such as lonafarnib and tipifarnib advanced to phase III clinical trials. Unfortunately, however the developed inhibitors failed to demonstrate efficacy in RAS-driven tumours, a finding that was later rationalized by the compensating action of other lipidation enzymes in particular prenyltransferases (Whyte et al. 1997).

Direct targeting strategies for RAS have been hampered by the absence of druggable binding pockets (Buhrman et al. 2011). However, a number of weakly binding fragments have been identified (Wang et al. 2012) (Fig. 4). Structural analysis revealed that all crystallized fragments bind to a pocket adjacent to the switch I/II regions. Indeed, the identified fragments inhibited SOS-mediated nucleotide exchange and prevented RAS activation by blocking the formation of intermediates of the exchange reaction (Maurer et al. 2012; Sun et al. 2012).

Other inhibitors have been developed based on the nonsteroidal, anti-inflammatory drug sulindac sulphide. Compounds of this class have been reported inhibiting the formation of the RAS–RAF complex and have been shown to inhibit proliferation of RAS-transformed cells. However, this compound class may have other off-target effects that may contribute to the observed phenotypes (Karaguni et al. 2002).

An interesting approach has been reported recently by Shokat and colleagues who recently reported small molecules that covalently and selectively bind to the

G12C mutant form of KRAS, a frequent mutation often found in non-small-cell lung cancer. The developed compounds blocked SOS1-mediated nucleotide exchange and decreased the binding of RAS to both BRAF and CRAF in G12C-mutated cell lines (Ostrem et al. 2013).

4 Example 3: Protein–Protein Interactions

Targeting protein has gained popularity after a number of highly potent inhibitors have been developed. The targeted interactions are however typically characterized by a well-defined binding site. For instance, interaction inhibitors that disrupt the binding of MDM2 (murine double minute 2) and MDMX to the tumour suppressor p53 have now entered clinical testing in cancer (Vassilev 2007; Brown et al. 2009). MDM2 binds the p53 tumour suppressor protein with high affinity and negatively modulates its transcriptional activity and stability. Overexpression of MDM2 is frequently found in tumours leading to impairment of p53 function. Thus, it has been hypothesized that inhibition of MDM2–p53 interaction can stabilize p53 and may offer a pharmacological strategy restoring p53 function in MDM2 overexpressing tumours.

Recent structural studies showed that a new class of dual MDM2/MDMX inhibitors block the binding of MDM2 and MDMX to p53 by stabilizing MDM2/MDMX homo- and heterodimerization occluding the p53 binding pocket (Graves et al. 2012).

A number of protein–protein interaction domains that selectively recognize sequences containing post-translational modifications have recently emerged as interesting targets for the development of inhibitors. The targeted interaction modules comprise in particular members of the so-called epigenetic reader domain family which include acetyl-lysine-dependent bromodomains (Muller et al. 2011; Filippakopoulos and Knapp 2014) as well as readers of methyl-lysine or methyl-arginine containing sequences such as PHD zinc finger domains and the Royal family of reader domains, which is composed of Tudor, MBT, PWWP and chromodomains (Herold et al. 2011a; James et al. 2013a). Several members of this family of protein interaction modules have good predicted druggability, and several inhibitors have been developed in particular for acetyl-lysine-dependent bromodomains (Vidler et al. 2012; Santiago et al. 2011). A shared feature of epigenetic reader domains that makes these protein interaction modules attractive targets is the observation that the interaction with their specific recognition sites is usually weak and localized to a binding pocket of suitable size for inhibitor development. Typically K_{Ds} of reader domain interactions are in the low μM regions suggesting that protein interactions mediated by these domains can be easily inhibited by low molecular weight inhibitors. In addition, lysine acetylation neutralizes the charge of the lysine side chain resulting in aromatic and hydrophobic binding sites. Indeed, fragment-based screening approaches identified several diverse chemotypes suggesting excellent druggability of bromodomains (Vidler et al. 2013).

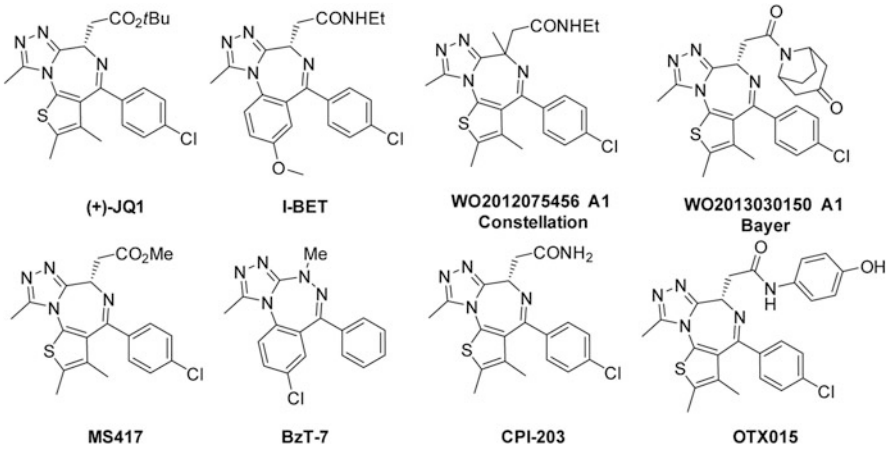

Fig. 5 Examples of benzodiazepine- and thienodiazepine-type BET inhibitors

The first bromodomain inhibitors appeared in the patent literature and were developed targeting the BET family (Miyoshi et al. 2009; Adachi et al. 2006). These inhibitors belong to the thieno-triazolo-1,4-diazepines which showed strong growth inhibitory activity on a panel of cancer cell lines. The disclosure of thieno-triazolo-1,4-diazepines as BET inhibitors led to the development of the thieno-triazolo-1,4-diazepines JQ1 (Filippakopoulos et al. 2010) (Fig. 5). In parallel, GSK discovered the benzo-triazolo-1,4-diazepine class of BET inhibitors (I-BET) using a combination of phenotypic screens and chemoproteomics (Chung et al. 2011; Nicodeme et al. 2010). Selectivity screening showed that benzo- and thienodiazepines are highly selective for BET bromodomains. Interestingly, the introduction of a stereo centre at the diazepine ring yielded a highly potent (S) enantiomer, (+)-JQ1, whereas the (R) enantiomer, (−)-JQ1, is inactive and may be used as a negative control compound. Crystal structures with BET bromodomains showed that the methyl-triazol ring served as an acetyl-lysine mimetic moiety and formed the canonical hydrogen bond with the conserved asparagine (N140 in BRD4(1)) or analogue residues in other BET family members (Filippakopoulos et al. 2010; Matzuk et al. 2012). The strong anti-proliferative effects of JQ1 and I-BET in cancer and the anti-inflammatory properties of these agents prompted the development of a number of similar benzodiazepine and thienodiazepine molecules which all include either modification on the ester/amid linkage (Zhang et al. 2012) or substitutions in the diazepine ring, which led for instance to benzotriazepines (Bzt-7) in which the asymmetric carbon was replaced by a nitrogen (Filippakopoulos and Knapp 2014; Filippakopoulos et al. 2012; Knapp and Weinmann 2013).

Novel acetyl-lysine competitive ligands have been developed based on fragment hits. In particular based on the 4-phenyl 3,5-dimethyl isoxazole fragment, a number of isoxaxoles have been developed as potent BET inhibitors (Hewings et al. 2011),

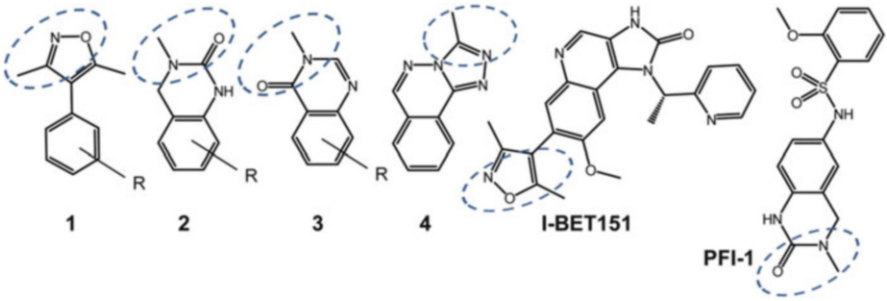

Fig. 6 Acetyl-lysine mimetic inhibitors of bromodomains. General scaffolds: (**a**) isoxazoles, (**b**) 3-methyl-dihydroquinazolin-2-one, (**c**) 3-methyl-quinazolin-4-one and (**d**) 3-methyl-triazolophthalazine. Developed high affinity inhibitors: I-BET151 and PFI-1. Acetyl-lysine mimetic moieties are highlighted by a *dashed circle*

most notably the highly potent panBET inhibitor I-BET151 (Dawson et al. 2011). Quinazolinone scaffolds (2-one or 4-one) have also been developed resulting in the panBET inhibitor PFI-1 (Picaud et al. 2013; Fish et al. 2012). Co-crystal structures confirmed the acetyl-lysine mimetic binding mode of the quinazolinone head group of PFI-1 which forms two hydrogen bonds with the conserved Asn140 in BRD4 (1) as well as a water-mediated hydrogen bond to the conserved tyrosine Tyr97 (Picaud et al. 2013).

BET bromodomain inhibition has recently been described as a frequent off-target activity of kinase inhibitors (Ciceri et al. 2014; Ember et al. 2014). A screen carried out on clinical kinase inhibitors revealed a number of inhibitors with potent BET activity suggesting that dual kinase/bromodomain inhibitors could be developed. The frequent hit rate in inhibitor screens and the large number of potent inhibitors that have been developed since the discovery of the triazolodiazepine-type inhibitors certify the excellent druggability of BET bromodomains (Fig. 6).

A number of potent bromodomain inhibitors have also been developed outside the now well-studied and well-explored BET family. Modification of the benzodiazepine scaffold led to the development of promiscuous bromodomain inhibitors (Bromosporines) that show broad spectrum activity targeting in particular BET family members, TAF1, CECR2, BRD7 and BRD9 and the BRPF family (Fig. 7). Selective inhibitors have been developed for bromodomains present in the histone acetyl transferases CREBBP/EP300 (I-CBP112 and CBP30) (Hay et al. 2014), BAZ2A/B (GSK2801 and BAZ2-ICR), BRPF1B (PFI-4) and panBRPF (OF1, NI-C-057), BRD7/9 (LP99) and panSMARCA/PB1(5) (PFI-3) (see http://www.thesgc.org/chemical-probes/epigenetics). The currently available probes represent a good coverage of chemical tool compounds for this family of epigenetic effector domains and demonstrate the feasibility targeting these interaction domains that have not been considered as druggable targets a few years ago. However, if any of the published inhibitors will be developed into an approved pharmaceutical remains to be shown. Clinical trials on BET inhibitors have been initiated recently.

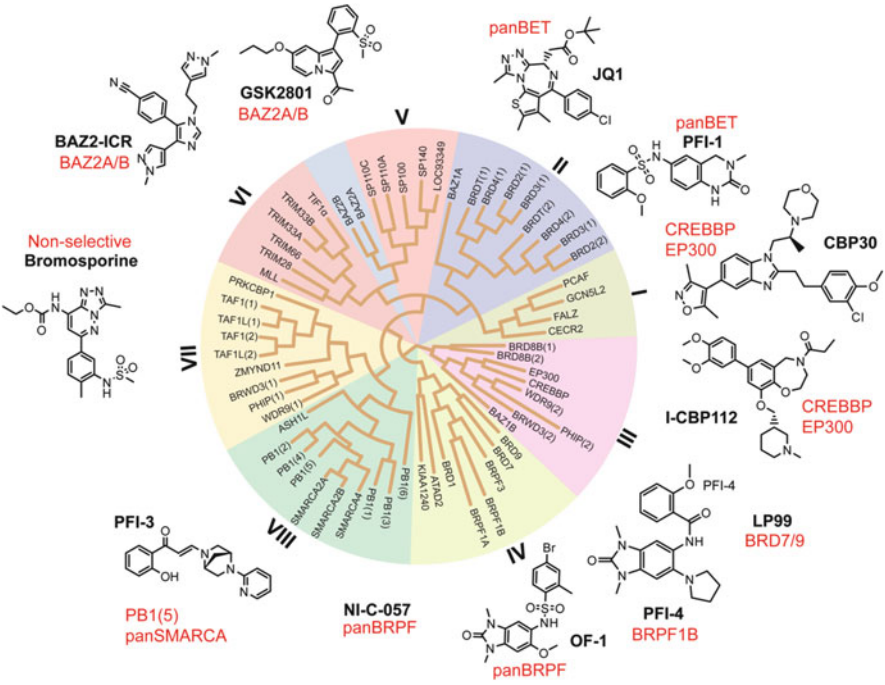

Fig. 7 Bromodomain chemical probes. Shown is a phylogenetic tree (*centre*) and the currently available chemical probes developed by the SGC (http://www.thesgc.org/chemical-probes/epigenetics). Targets that are inhibited are shown in *red*. The structure of the BRD9/7 inhibitor LP99 and the panBRPF inhibitors is currently undisclosed

Methyl-lysine/arginine reader domains have also recently been targeted but the diversity of this large protein interaction module family and the binding site properties of most methyl-lysine/arginine readers render chemical probe/inhibitor development more challenging. One of the first methyl-lysine epigenetic reader domains that have been targeted was L3MBTL3, a member of the malignant brain tumour (MBT) family of chromatin-interacting transcriptional repressors. Optimization of weaker starting points (Herold et al. 2011b) led to the discovery of UNC1215 as a potent and selective chemical probe for L3MBTL3 (James et al. 2013b). This chemical probe binds with a K_D (dissociation constant) of 120 nM and has excellent selectivity for the targeted reader domain. Biophysical analysis revealed that UNC1215 has a methyl-lysine competitive binding mode, effectively displacing dimethyllysine-containing peptides from the L3MBTL3 binding site.

Recently, another potent methyl-lysine reader domain has been reported. OICR-9429 is a selective chemical probe for WDR5, a protein that is present in several chromatin regulatory complexes including the MLL1 (mixed lineage leukaemia 1) complex. OICR-9429 binds to WDR5 with K_D values of 24 nM (Biacore) and 52 nM (ITC) and was found to be more than 100 selective over other chromatin 'reader' domains, methyltransferases and other non-epigenetic targets (Fig. 8).

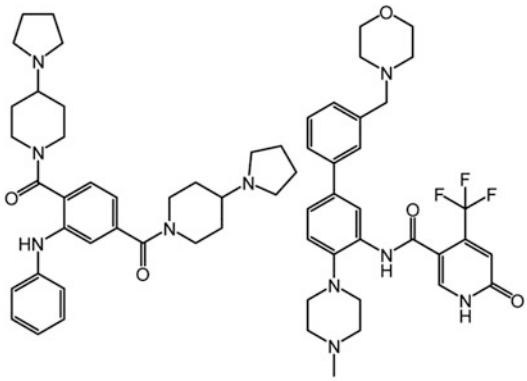

Fig. 8 Chemical probes targeting methyl-lysine reader domains

A recent druggability analysis of the methyl-lysine/arginine family of reader domains suggested that there are many additional opportunities for the development of selective and potent inhibitors. Improved druggability of these domains may be achieved targeting adjacent pockets. For instance, the methyl-lysine binding site present in CBX is largely extended by a channel that harbours flanking peptide sequences. The ankyrin repeat protein GLP is another example for extended binding sites that lead to improved druggability scores. A mono- or dimethyl containing sequence is anchored into a central cavity containing a typical aromatic cage. The duggability of this pocket is poor ($D_{score} = 0.64$; PDB code, 3B95); however, considering two adjacent pockets less than 7 Å apart improved the druggability score to $D_{score} = 0.98$ (Herold et al. 2011a). Methyl-lysine reader domains are often mutated in cancer and genetic diseases suggesting that targeting these domains may be beneficial for a number of disease applications.

Recent reports strongly suggest that many protein–protein interactions that are mediated by localized interactions are highly druggable and that potent and bioactive inhibitors can be identified. Some of the developed inhibitors are now also tested in phase I/II clinical trials, and it is likely that many more protein–protein interaction inhibitors will be identified and developed in the near future. The development of bioactive inhibitors that target larger interfaces remains however very challenging, and this area would require additional intensive research efforts before potent bioactive inhibitors will be identified and tested clinically.

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In Vivo Target Validation Using Biological Molecules in Drug Development

Derek S. Sim and Katalin Kauser

Contents

Abstract

Drug development is a resource-intensive process requiring significant financial and time investment. Preclinical target validation studies and in vivo testing of the therapeutic molecules in clinically relevant disease models can accelerate and significantly de-risk later stage clinical development. In this chapter, we will focus on (1) in vivo animal models and (2) pharmacological tools for target validation.

Keywords

Bleeding disorders · Gene therapy · Gene-editing · Hemophilia · Monoclonal antibodies · Mouse models · Non-mammalian models · Recombinant proteins · Target validation

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1 Ideal Animal Disease Model for Target Validation

An ideal animal disease model for target validation would be a model that recapitulates the disease phenotype, shares the same pathophysiology as human, and responds to existing human therapies in a manner similar to patients. Animal models that faithfully resemble the disease pathophysiology are invaluable for the characterization of the mechanism of action, pharmacokinetics, pharmacodynamics, biomarkers, safety, and toxicity of future therapies. Such animal models could also help to predict the human dose prediction for clinical trials. While these principles are obvious, the available animal models often have limitations and do not meet these ideals or mimic closely enough the human diseases. Far too often, molecules that provided impressive efficacy in preclinical models fail in clinical trials. This could be due to the preclinical disease model species being only distantly related to human. It could also be due to that inappropriate endpoints have been used for target and efficacy evaluation in the chosen models. Another possibility for the failed translatability from experimental models to the clinic could also be flawed clinical trial designs. Regardless of these potential hindsights, in vivo target validation with predictive animal models is a crucial part of the drug development process. If performed well, with careful consideration to minimize obtaining misleading information, in vivo disease models represent significant value for discovery and development of new treatment options for patients.

To initiate in vivo target validation, the first decision to be made is to select a validation approach. In this chapter, we will focus on (1) in vivo animal models and (2) pharmacological tools for target validation.

2 In Vivo Models for Target Validation

2.1 Mammalian Models

2.1.1 Wild-Type Mice

Wild-type mouse strains are indispensable tools for medical research and drug development. Disease phenotypes can be induced in these mice by genetic, pharmacological, or surgical means. C57BL/6, FVB/N, BALB/c, C3H/He, and 129Sv are some of the commonly used wild-type mouse strains. However, it is important to recognize there could be differences among these different strains that could affect one's target validation effort.

For example, special attention should be paid when selecting mouse strains for studying Th1 and Th2 responses. C57BL/6 and BALB/c mice are, respectively, regarded as Th1- and Th2-dominant mouse strains. T cells from C57BL/6 mice preferentially produce Th1 cytokine with high interferon-γ (IFNγ) and low interleukin (IL)-4. In contrast, BALB/c mice produce Th2 cytokine production with low IFNγ and high IL-4 (Mills et al. 2000). Similar T-cell responses were observed when these mouse strains were evaluated in Leishmania major infection studies (Heinzel et al. 1989; Scott et al. 1988). Therefore, for the purpose of validating

immunological targets, one needs to take into consideration the Th1/Th2 balance in mice of different genetic background (Hsieh et al. 1995; Stewart et al. 2002).

An example on the mouse strains of different genetic background contributing to differences in hemostasis can be found in the platelets of FVB mice. While the expression of major receptors such as integrin αIIb, integrin β3, glycoprotein (GP) Ibα, or GPVI on platelets from FVB mice is similar to those from C57BL/6, BALB/c, C3H/He, and 129Sv, the expression level of integrin α2 on FVB was approximately 50% lower than as platelets from the other strains. This difference leads to a marked reduction in platelet aggregation and a longer lag phase when activated by collagen (Li et al. 2004).

These examples highlight the importance in performing a thorough literature search on the pathophysiology mechanism in the different strains of mice and selecting the most relevant background strain before in vivo validation. An online tool, Mouse Phenome Database (MPD), which contains database of strain characterization data on the phenotype and genotype of the different mouse strains, is also a valuable tool that could help investigators to identify mouse strains appropriate for their studies (Bogue and Grubb 2004; Grubb et al. 2004, 2014). These readily available literature and data should be consulted and considered as one of the first steps for in vivo target validation using mouse models.

2.1.2 Genetically Engineered Mouse Models

Genetically engineered mouse models (GEMMs) have been a powerful tool for studying human disease and for drug development (Cook et al. 2012; Kucherlapati 2012). GEMMs have not only helped elucidating the pathophysiology of human diseases; these models are also indispensable for in vivo target validation.

When a mouse model is needed within a short period of time, transgenic mice, generated by inserting a bacterial or viral vector into a fertilized mouse egg, can be an option to generate the required genetic model (Gordon et al. 1980). In transgenic animal, the transgene usually integrates into one or more loci. This is a relatively quick approach in establishing a genetically engineered animal, but one needs to be aware of the possible risk that transgene may be inserted into locus with critical functions. As a result, the transgenic mice would develop additional confounding phenotypes. Therefore, it is advisable to evaluate simultaneously several independent lines of mice with the same transgene during the in vivo target validation stage.

Mutagenesis by homologous recombination in embryonic stem (ES) cells (Koller et al. 1989) is a targeted approach for inactivating, overexpressing, and humanizing the genes of interests. Thousands of genetic knockout, knockin, and humanized strains have been generated by this approach. A knockout mouse with targeted gene disruption is used to validate the in vivo function of the gene. Additionally, such a knockout model can be used for evaluating pharmacological agents. While knockout mouse technology is a valuable tool, the disruptions of some genes are developmentally lethal. As a result, some genetically altered embryos cannot develop into adult mice for the purpose of determining a gene's in vivo function after birth. In some instances, a gene may serve a different function in adults than in developing embryos. These problems can be circumvented with technologies developed for generating inducible and tissue-specific knockout

Table 1 Animal models with bleeding disorders for in vivo target validation

	Weight	Mutation site	Spontaneous bleeding
Genetically engineered models			
Hemophilia A mouse (Bi et al. 1995)	20–30 g	Exon 16 or 17 deletion	Rare
Hemophilia A pig (Kashiwakura et al. 2012)	20–30 kg	Exon 16 deletion	Common and severe
Hemophilia B mouse (Lin et al. 1997)	20–30 g	Promoter to exon 3 deletion	Rare
vWD mouse (Denis et al. 1998)	20–30 g	Exon 4–5 deletion	10% in pups, rare in adults
Larger congenital disease animal models			
Hem A rat (Booth et al. 2010a, b)	200–300 g	Leu176Pro	20%, tarsal joint bleeds
Hem A dog (Giles et al. 1982; Brinkhous and Graham 1950; Graham et al. 1949)	10–30 kg	Intron 22 inversion	Common, joint bleeds
Hem A sheep (Neuenschwander et al. 1992; Porada et al. 2010)	70–75 kg	Exon 14 stop codons	Common, joint bleeds
Hem B dog (Mauser et al. 1996; Evans et al. 1989; Mustard et al. 1960)	15–20 kg	772–776 nucleotide deletion and a C to T transition at 777 (Mauser et al. 1996)	Common, joint bleeds
		G1477A (Evans et al. 1989)	
vWD dog (Haberichter et al. 2005)	8–10 kg	C255 deletion in exon 4	Mucosal surface
vWD Pig (Fass et al. 1979)	60 kg	To be defined, likely point mutation (Bahou et al. 1988)	Mucosal surface

animals (Furth et al. 1994; Kistner et al. 1996). These technologies allow the mouse to develop and mature normally before the gene of interest being ablated.

The first hemophilia A mice with the targeted deletion of coagulation factor VIII was created in 1995 by the disruption of exon 16 or exon 17 (Bi et al. 1995). Hemophilia B mice was created in 1997 by disrupting the promoter through exon 3 region of factor IX (Lin et al. 1997). Similar to the human conditions, the deficiency of factor VIII and factor IX in these mice leads to severe bleeding phenotype upon injury (Table 1). These hemophilia mouse models have been used extensively to validate the PK, PD, and efficacy of hemophilia therapies, including novel therapeutics that could provide extended duration of action (Metzner et al. 2009; Peters et al. 2010; Ostergaard et al. 2011; Dumont et al. 2012; Stennicke et al. 2013; Pastoft et al. 2013; Mei et al. 2010) and enhanced efficacy (Lin et al. 2010; Leong et al. 2015). These mouse models have also enabled gene therapy studies (Crudele et al. 2015; Wang et al. 1997; McIntosh et al. 2013) and different delivery routes (Peng et al. 2010; Brooks et al. 2013).

2.1.3 Larger Disease Animal Models

While GEMM is powerful, it is sometimes limited by the fact that mice are being distantly related to humans. As a result, there could be low homology in some of the drug targets as well as differences in pathophysiology in comparison to patients. Additionally, the small body size of mice also becomes a limitation for sampling of biological specimens, monitoring of vital signs, and performing certain surgical procedures. Therefore, alternative and larger species can be desirable as disease models for target validation.

For the study of bleeding disorders, a collection of larger disease models, most of them congenital, are available for investigators (Table 1). Hemophilia A rat (Booth et al. 2010a, b), hemophilia A dog (Giles et al. 1982; Brinkhous and Graham 1950; Graham et al. 1949), hemophilia B dog (Mauser et al. 1996; Evans et al. 1989; Mustard et al. 1960), hemophilia A sheep (Neuenschwander et al. 1992; Porada et al. 2010), and hemophilia A pig (Kashiwakura et al. 2012) have been described in literature and used for gene and cell therapy studies. Dogs (Haberichter et al. 2005) and pigs (Fass et al. 1979) with von Willebrand's disease bleeding disorders have also been described. Many of these animals are more closely related to humans and may be related better to the clinical situation.

2.2 Nonmammalian Models

For the purpose of in vivo target validation, nonmammalian species could also be attractive options. Due to the short reproductive and life cycles of zebrafish (Spence et al. 2008) and *Caenorhabditis elegans*, these species could be cost-effective models that provide rapid turnaround of data. Additionally, their genomes have been sequenced, and the homology to human equivalent genes has been established for these simple organisms to facilitate in vivo target validation (Howe et al. 2013; *C. elegans* Sequencing Consortium 1998).

2.2.1 Zebrafish

Zebrafish is a small freshwater fish native to Pakistan and India (Spence et al. 2008). It has become an important model organism for studying vertebrate development and gene function.

Transgenic zebrafish has been used as models of cancer (Liu and Leach 2011), cardiovascular diseases (Drummond 2005), and immune diseases (Novoa and Figueras 2012).

2.2.2 *C. elegans*

Caenorhabditis elegans is a small roundworm with 959 somatic cells with a life cycle of 3–5 days (Felix and Braendle 2010). It is the first multicellular organism to have its genome sequenced (C. elegans Sequencing Consortium 1998). Human has 74% of its genome sequence matching *C. elegans* and shared a significant number of biological pathways. Transgenic worms have been generated to evaluate the role of specific mutations in human disease pathophysiology. These properties have allowed *C. elegans* to become an attractive model platform for in vivo drug target

validation and compound screening for drug discovery in indications such as neurological diseases (Alzheimer's, Parkinson's, and Huntington's diseases) (Li and Le 2013), and cancer (Kirienko et al. 2010).

3 Biological Tools for Target Validation

3.1 Biologic Approaches

Monoclonal antibodies and recombinant proteins are important biologic tools for in vivo target validation. Depending on the targets, antagonistic or agonistic monoclonal antibodies could be used for target validation. Recombinant proteins could also have activating or inactivating properties depending on the specific targets or pathways being interrogated.

Validation by biologics is possible of targets, which reside in a physiological location accessible by the administered biologics, for example, soluble targets in systemic circulation, cell surface receptors, and extracellular tissue compartment targets. To validate a soluble target, the plasma concentration of the target and the affinity of the biologics towards the target need to be determined beforehand in order to ensure that the validation study is performed with a dose that saturates the target in question. For the validation of cell surface target, attention should be paid on whether the surface target becomes released into circulation as part of the physiological or disease process or gets internalized. The soluble form of the target can act as a sink and reduce the amount of biologics available for engaging the target on cell surface.

The pharmacological properties of the biologics need to be determined before the initiation of in vivo target validation study. The plasma threshold concentrations of biologics should be obtained with in vitro binding and potency studies to predict the level, which can provide efficacy. Additional pharmacokinetic studies will help to design and establish the plasma concentration needs to be attained for in vivo studies. Furthermore, the clearance rate and duration of action of the biologics should also be characterized with the help of these pharmacokinetic and pharmacodynamics studies. These information will guide the decision of the dosage and dosing regimen of the biologics in animals for in vivo target validation study.

While the use of biologics is a valuable approach for in vivo target validation, this process is sometime hampered by the development of immunogenicity against the biologics. This is especially true for longer-term validation studies in which animals receive multiple doses of the biologics. This could lead to the development of antibodies which could neutralize the activity and the efficacy of the biologics. Therefore, the monitoring of antibody development towards the biologics during the validation is necessary, especially in studies where no efficacy was observed. One way to reduce the risk of immunogenicity development is to use species-matching biologics, e.g., mouse monoclonal antibody in a mouse model but not a rat model.

Another potential hurdle of using biologics for target validation is the lack of existing tool molecules of high quality and high specificity to the target of interest.

In this situation, new biologic molecules need to be generated de novo. However, this is a very time-consuming process. Therefore, one should consider whether validating upstream or downstream targets within the same pathway using existing tool compounds to achieve partial validation of the target and pathway of interest would be an acceptable alternative.

3.2 Genetic Approaches

Genetic approaches are complementary strategies for evaluating targets difficult for using biologics due to the lack of accessibility of targets or the availability of biologic tool compounds. There has been a growing collection of genetic approaches that investigators can choose from for validating drug targets. These methods include but are not limited to adeno-associated virus (AAV), lentivirus, RNA interference (RNAi), zinc-finger nuclease (ZFN), transcription activator-like effector nucleases (TALEN), meganuclease, and CRISPR-Cas9 technologies.

Adeno-associated virus (AAV) can be used to knock out gene expression or to introduce genes in mice for in vivo target validation. AAV has been used to express a short hairpin RNA (shRNA) in the hypothalamus to generate a mouse model of obesity (Garza et al. 2008). AAV-induced knockout of vascular endothelial growth factor (VEGF) in the lung generated a model of emphysema (Tang et al. 2004). The introduction of genes by AAV in mice can also replace the use of therapeutic proteins for target validation. This can be achieved by introducing cDNAs into recombinant AAV vectors and injecting mice with rAAV. AAV represents a genetic approach for in vivo target validation. Similarly, lentiviral vectors have also been combined with RNAi approaches to achieve gene knock down for target validation (Rubinson et al. 2003; Ventura et al. 2004). However, it is important to note that gene disruption by RNAi approaches can be variable and short-lived. Therefore, phenotypic observations need to be interpreted with caution.

On the other hand, genome editing approaches such as ZFN (Urnov et al. 2010), TALEN (Sun and Zhao 2013), meganuclease (Menoret et al. 2013), and CRISPR-Cas9 (Platt et al. 2014) directly modify the DNA with nucleases to generate permanent modifications of the target genes within the genome for in vivo gene function validation. The overall concept of genome editing is to introduce a double-stranded break (DSB) within the target gene by directing the nuclease to the specific loci with complementary sequences. Following DSB, disruption of the gene or introduction of new gene sequence can be performed. There has been a rapid adoption of these technologies in the recent years. Among these technologies, CRISPR-Cas9 is considered to be a major advance for sequence-specific genome editing due to potential advantages of improved efficiency, ease of design, and potentially reduced off target editing over earlier genome editing approaches. Overall, gene editing approaches have a higher efficiency than generating knockout model by homologous recombination (Gaj et al. 2013). The advancement of these genetic techniques has offered new options for in vivo target validation.

4 Challenges in Target Validation and Clinical Translatability of Preclinical Model

While a variety of tools are available to enable in vivo target validation, it remains a challenging process as evidenced by the number of failed clinical trials (Hay et al. 2014). This is because animal models often do not fully recapitulate the disease phenotype or share the same pathophysiology as patients. The targets in the animal models may have a different tissue expression and distribution than in humans. Also, the pathophysiological pathways in patients could be evolutionarily diverged from the animal models and serve a different mechanism of action. Therefore, it is most desirable to validate a target in at least two species with different approaches to gain further confidence in clinical translatability before entering the resource-intensive clinical development phase of drug development.

4.1 Translatability of Preclinical Animal Models: Hemophilia Mice as Examples

Hemophilias A and B are characterized by the deficiency of coagulation factors VIII and FIX, respectively, in patients. The deficiency in these coagulation factors leads to complications such as joint bleeds, intramuscular bleeds, and sometimes intracranial bleeds. Bleeding into joints eventually leads to inflammation. Overtime, patients develop joint arthropathy and severe limitation to mobility. In hemophilia clinical trials, the annualized bleed rate in patients is used as the efficacy endpoint to evaluate the efficacy of a therapy. Genetically engineered hemophilia mouse models have been invaluable for hemophilia research and drug development. Coagulation factor VIII (FVIII) and factor IX (FIX) have been deleted in mice to generate hemophilia A and hemophilia B models, respectively. Both mouse models suffer from severe bleeding upon injury. All new drugs developed for hemophilia are tested in these models. While these hemophilia mice provided successful clinical translatability in patients, there are significant differences between clinical observations in patients and the actual disease phenotype in mice. Unlike patients, hemophilia mice very rarely develop spontaneous joint or soft tissue bleeds (Bi et al. 1995). This observation is likely due to the small size of mice exerting significantly lower load-bearing stress to the joints. Therefore, an acute bleeding model induced by tail amputation has been used as a surrogate model to assess impact of the therapy on bleeding. The acute tail amputation is a relative simple model for evaluating the efficacy of a coagulation factor (Mei et al. 2010; Elm et al. 2012). However, it is rather insensitive, needing $\geq$50% FVIII to correct bleeding phenotype, a level significantly higher than the 1% FVIII threshold accepted to be required for prophylaxis in humans. Also, the acute tail cut model also does not evaluate efficacy for recurrent rebleeds. Therefore, for a more faithful recapitulation of human physiology, more sensitive injury model such as the tail vein transection model and the saphenous vein injury model has been developed. In comparison to acute tail amputation model, only a vein is injured

in the tail vein transection model and saphenous vein model. As a result, the sensitivity of these models is as low as a few percent of FVIII, a level relevant to prophylaxis in humans and better suited for testing the new generation of long-acting coagulation molecules (Mei et al. 2010; Pastoft et al. 2012). With these specific efficacy models, the hemophilia mouse models have proven to be a predictive model with high clinical translatability for patients.

5 Conclusion

In vivo target validation with predictive animal models is a crucial part of the drug development process. A successful preclinical target validation study can significantly de-risk a clinical development program. Therefore, the selection of an animal model and tools to be used for in vivo validation must be performed with careful deliberation on the experimental design and a full understanding of the pharmacology of the tool molecules used in the experiments in order to have a model system that closely resembles the human disease condition.

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High-Throughput Synthesis of Diverse Compound Collections for Lead Discovery and Optimization

C. Rademacher and P.H. Seeberger

Contents

Abstract

Small-molecule intervention of protein function is one central dogma of drug discovery. The generation of small-molecule libraries fuels the discovery pipeline at many stages and thereby resembles a key aspect of this endeavor. High-throughput synthesis is a major source for compound libraries utilized in academia and industry, seeking new chemical modulators of pharmacological targets. Here, we discuss the crucial factors of library design strategies from the perspective of synthetic chemistry, giving a brief historic background and a summary of current approaches. Simple measures of success of a high-throughput synthesis such as quantity or diversity have long been discarded and replaced by more integrated measures. Case studies are presented and put into context to

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highlight the cross-connectivity of the various stages of the drug discovery process.

Keywords
Automated synthesis · Drug design · High-throughput synthesis · Library design

Abbreviations

ADMET	Absorption, distribution, metabolism, excretion, and toxicity
BIOS	Biology-oriented synthesis
DCR	Divide, couple, and recombine
DEL	DNA-encoded libraries
DOS	Diversity-oriented synthesis
ELISA	Enzyme-linked immunosorbent assay
MAPK	Mitogen-activated protein kinase
PS-SPCL	Positional-scanning synthetic-peptide combinatorial library
TOS	Targeted-oriented synthesis
VEGFR2	Vascular endothelial growth factor receptor 2

1 Introduction

Identification of biologically active small molecules is a multidisciplinary task at the heart of chemical biology and drug discovery (Bleicher et al. 2003). The cross-connectivity between hit generation, lead identification, optimization, and the evaluation of compounds in a biologically relevant environment renders this a challenging task. Modern drug discovery approaches have faced a decline in therapeutic output over the last decade (Carney 2005; Walters et al. 2011). While genomics and proteomics have identified many potential drug targets, the identification of innovative therapeutic agents has stalled (Carney 2005). The stark contrast between the 300–500 pharmaceutically targeted proteins and estimated 30,000 genes in the human genome underscores this discrepancy (Bleicher et al. 2003). Therefore, new avenues have to be taken, integrating efforts from drug companies, chemistry, and software development.

In this race for new drug leads, high-throughput synthesis is of fundamental importance as it fuels the pipeline with new chemical entities at an early stage of research. Moreover, rapid generation of compounds contributes significantly to lead optimization processes further along the pipeline. Taking into account the complexity of the development pipeline, synthesis is at the heart of the discovery process. The initial strategic planning of a screening library has changed since it became clear during the last 20 years that the size of a library is not as an important determinant as initially thought. During the mid-1990s, large compound collections

dominated the field. Libraries included many chemically feasible or almost all commercially available molecules. However, low hit rates and difficulties at later stages of the discovery process such as unwanted ADMET properties (Hodgson 2001) and failed optimization schemes in medicinal chemistry (Walters et al. 2011) made many laboratories rethink their screening strategies to focus on the selection of compounds for their screening libraries (Schreiber 2009). Consequently, new high-throughput synthesis schemes were developed based on these experiences.

Diversity has emerged as a common denominator in modern library design (Fig. 1). Without any prior knowledge of the drug target, chemical libraries have to be diverse in order to probe any given target with an increased likelihood of finding biologically active molecules (Tan 2005). And even with information on privileged scaffolds or known ligands, screening diverse libraries offers an excellent opportunity for scaffold hopping toward novel drug candidates (Zhao 2007). Unfortunately, the definitions found for diversity in the context of small molecules are diverse themselves (Stumpfe and Bajorath 2011). For instance, a broad range of biological outcomes can define the functional diversity of compound selections. Alternatively, structural diversity can be described by the variety 3D arrangement of functional groups around diverse chemical scaffolds. Nevertheless, despite the fuzzy definition of diversity, the identification of a new chemical agent that acts as a modulator of a defined target, which elicits the desired therapeutic outcome, one has to realize that at some point all molecules have to be synthesized.

With progress in instrumentation, screening methodologies, and theoretical library design strategies, the demands on chemical synthesis have risen tremendously. Notably, measures to assess the quality of a synthetic effort are

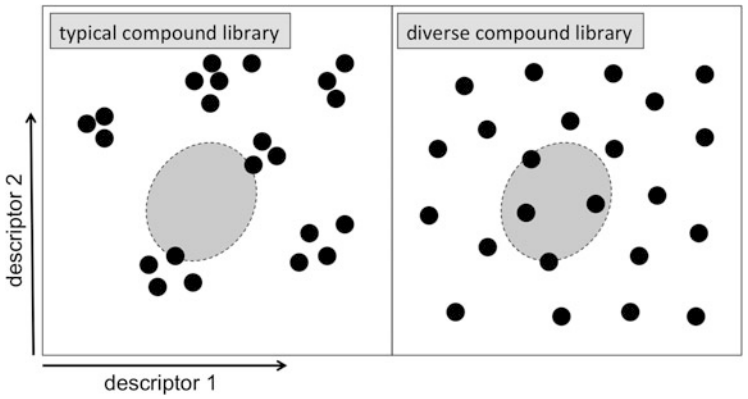

Fig. 1 Schematic presentation of the diversity of a screening library. Clusters of closely related entities determine the composition of a typical compound library (*left*). These clusters originate from the similarity of the underlying synthetic chemistry (Nadin et al. 2012). Thereby, the chances to actually occupy the activity island of a drug target (*gray shaded area*) are significantly decreased. On the other hand, a chemically diverse set of compounds occupies a larger fraction of the chemical space (*right*), promoting lead identification (Patterson et al. 1996). Additionally, valuable information for progressing hits into leads emerges from screening a diverse library

plentiful and truly not limited to the final library itself. This final product has to be diverse but still compatible with the activity assay. Therefore, high rates of false positives such as aggregators should be avoided above all (McGovern et al. 2002). Moreover, fluorescent molecules should not be included when the detection during screening is fluorescence-based. Another important aspect of the library design is the optimization of the resulting hits from the screening. Sufficient synthetic room for medicinal chemistry should be provided already at the stage of library construction (Kodadek 2010). Finally, conflicts with intellectual property rights, the price for scale up of the synthesis, and compliance with ADMET should be considered before a compound collection is established.

In the following, we will summarize a selection of past and recent developments in high-throughput synthesis in order to give an overview, knowing that a comprehensive description of such a vivid field is impossible. From past experience, many philosophies have evolved and influenced the high-throughput synthesis. Many chemical principles arose to fulfill the demands outlined above. So, in the end, the chemist has to pick his tools from a diverse set of approaches to achieve the specific goal in mind.

2 Principles of High-Throughput Synthesis in Lead Generation and Optimization

High-throughput synthesis impacts drug development by increasing the transition between target identification and lead development. The use of simple and robust chemistry is one fundamental requirement for the strategic planning of a synthesis scheme with sufficient throughput. Hence, progress in organic chemistry has always driven progress in high-throughput synthesis and provides grounds for technological advances (Galloway et al. 2010; Kodadek 2010; Schreiber 2009). Following a series of synthetic steps, a screening library has to be generated in quantities and purities that allow characterization, screening, and follow-up studies. Besides sufficient diversity in compound selection, high-throughput chemistry also influences later stages of the lead discovery and optimization process. In this respect, high-throughput synthesis is no different than other elements of the drug discovery pipeline but has to become part of an integrated thought process.

Three basic steps determine the strategies for high-throughput synthesis being (1) the choice of the synthetic platform, (2) the starting scaffold, and (3) the diversification chemistry. Firstly, as the most fundamental choice, the chemistry is either performed in solution or on a solid support. Although many high-throughput chemistry campaigns favor solution phase methods nowadays, solid-phase chemistry has been the origin of combinatorial chemistry and has been the platform of choice for high-throughput synthesis methods in particular with respect to polymer-based compounds. Therefore, this review mainly focuses on this type of chemistry. The ease to drive reactions to completion, the ability to parallelize synthetic efforts, and finally purification of the products from excess of reagents and solvents are the major advantages of chemistry on solid support. Consequently,

the choice of solid support material and linker chemistry is crucial. Many different solid supports have been developed over the past two decades, which vary according to their reactivity, solubility, stability, swelling, and surface chemistry. Even though polystyrene resins are commercially available and have found application in many research labs, the choice of solid support still relates to the synthetic route desired and may require case-by-case adjustment. The same is true for the choice of linker chemistry. In general, the linker should be chemically resistant but specifically cleavable to release the product from the resin. Moreover, the cleavage should not result in any artifacts left on the scaffold. Many UV-cleavable linkers have been developed that fulfill these requirements.

Secondly, a starting scaffold has to be attached to the linker on the solid support. This step determines the ability to optimize and scale up the generation of analogs, when lead structures are identified. In this, the choice of scaffold significantly establishes the diversity of the library. It is important to emphasize that the definition of diversity already varies from approach to approach at this particular stage of the synthesis. Diversity can result from similar scaffolds presenting diverse appendages or diverse scaffolds presenting similar functional groups in different spatial arrangements. This then determines how much investment into synthetic chemistry will be done at an early stage of the high-throughput synthesis pipeline. There is a great variability in current approaches in this point ranging from no diversity of the scaffold to high structural complexity of the starting skeletons. Finally, the third aspect of strategic planning of a high-throughput synthesis comprises the further diversification chemistries of the scaffold, again restricted by the ADMET, price, ease, and robustness of the chemistry in mind.

3 Combinatorial Peptide Synthesis

A powerful combinatorial approach arose with the availability of solid-phase peptide synthesis developed by Merrifield in 1963 (Merrifield 1963). Geysen and Houghten opened a new area with their pioneering work in combinatorial peptide synthesis (Geysen et al. 1984; Houghten 1985). Initially designed for the synthesis of very large libraries, using methods such as “divide, couple, and recombine” (DCR) (Houghten et al. 1991), many other studies followed exploring the newly developing field of combinatorial chemistry. Even though the diversity of these libraries was restricted to peptide chemistry, significant contributions in the field of lead discovery were already made in these early days (Houghten et al. 1991). Choosing mesh-packets as a solid support, so-called tea bags, or polyethylene rods (or pins), many important questions in combinatorial chemistry were addressed (Houghten et al. 1991; Weiner et al. 1992). The idea of systematic analysis of peptide binding targets using combinatorial libraries arose, and these studies directly contributed to biology and immunology (Pinilla et al. 1992).

In a seminal positional-scanning synthetic-peptide combinatorial library (PS-SPCL) approach, peptide mixtures of approximately 3×10^{11} components were screened for their ability to be recognized as T cell epitopes, adding up to a total of 6.4×10^{12} decapeptides analyzed (Hemmer et al. 1998). In another

example, more than four trillion decapeptide sequences were screened against a monoclonal antibody to identify candidates with increased affinity (Pinilla et al. 1994). The approach was successful and peptides with affinities ten-times higher than the natural ligand were identified using competitive ELISA.

Alternative biological approaches toward peptide libraries arose with phage display techniques (Smith 1985), and the real strength of the synthetic approach exploring the chemical space of peptide libraries was discovered by introducing chemical transformations of the peptide backbone into the synthetic schemes. Small peptides are not very well suited as drugs because of their low stability and oral availability. As a result, the "Libraries from Libraries" approach was developed by chemical modification of sublibraries (Houghten 2000; Nefzi et al. 2004; Ostresh et al. 1994). One of the first examples, permethylation of a hexapeptide library, afforded around 40 million compounds as mixtures in solution (Ostresh et al. 1994). Soon afterward, the method included heterocycle synthesis, inspired by solid-phase peptide chemistry (Fig. 2). First performed by Leznoff and Rapoport, solid-phase heterocyclic chemistry really gained momentum when applied to the synthesis of benzodiazepine analogs some 20 years later by Bunin and Ellman (Bunin and Ellman 1992; Crowley and Rapoport 1976; Leznoff and Wong 1973; Pinilla et al. 2003; Wong and Leznoff 1973). The development of a sophisticated toolbox for chemical transformations such as acylations, alkylations, and reductions accelerated the diversification of the resulting compound mixtures.

The "Libraries from Libraries" approach results in large compound collections in a mixture-based format. This strategy requires complex deconvolution schemes of successive screening of smaller subsets for the identification of hits from these

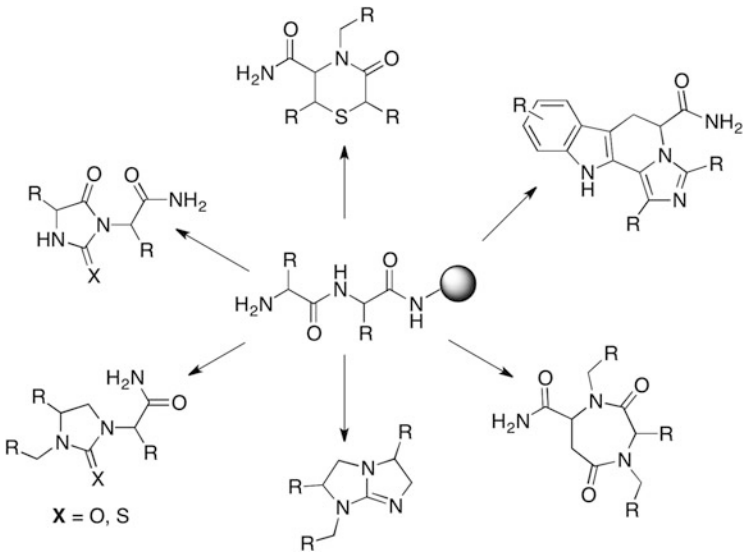

Fig. 2 Synthesis of heterocyclic compounds on solid support from dipeptides and acylated dipeptides as starting materials. Figure adapted from Houghten (2000)

mixtures. Therefore, fine-tuning of the mixtures must guide the combinatorial synthesis scheme taking into account expected hit rates, thereby minimizing later deconvolution procedures (Barnes and Balasubramanian 2000). Employing mixtures instead of defined sets of compounds (vide infra) anticipates a significant decrease in time between synthesis and screening. For the process of lead development, this rather pragmatic and straightforward collection of information from screening mixtures is proposed to be advantageous. Taking into account the massive size of these screening libraries, other techniques might not be capable of handling it.

A major advancement in the field of combinatorial synthesis of peptide-like structures came with the solid supported synthesis from peptide-like backbone structure without further chemical manipulation by Kodadek and coworkers (2009). Using *N*-substituted glycine units (peptoids), the diversity of side chain chemistries on a peptide-like backbone presentation was explored. The side chain extends from the main chain nitrogen rather than the alpha carbon and allows preservation of favorable peptide conformations, while at the same time peptoids are protease resistant. Peptoids have been described in 1992 (Simon et al. 1992), but early attempts failed due to limited monomer supply and the resynthesis of smaller pools during the deconvolution process (Zuckermann and Kodadek 2009). Recently, advances in oligo-*N*-alkylglycine chemistry and screening technology enabled inexpensive screening of large peptoid libraries. One success story is the discovery of high affinity binders of the vascular endothelial growth factor receptor 2 (VEGFR2) (Udugamasooriya et al. 2008) (Fig. 3). Peptoids coupled to fluorescent

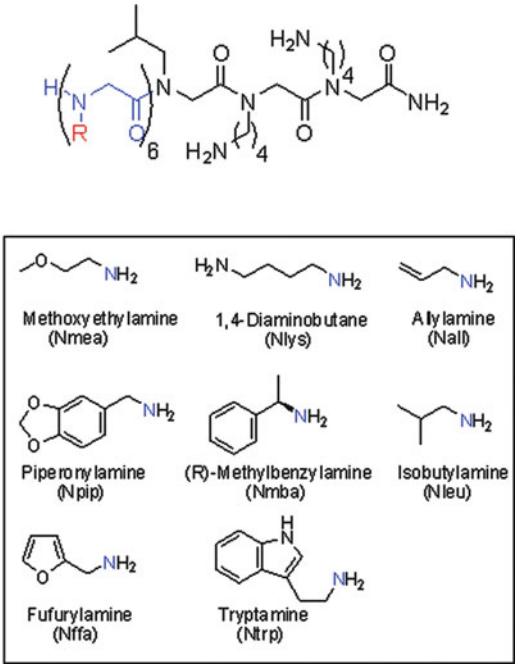

Fig. 3 Schematic representation of a peptoid library. General structure of a compound from a 250,000-membered peptoids library used by Udugamasooriya et al. (2008). The three *C*-terminal residues were fixed, while diversification was applied to the six N-terminal residues (drawn in *blue*). The main chain nitrogen is substituted with a combination of R-groups as depicted in the *box* (*blue nitrogens* represent the respective main chain nitrogen in the peptoids). Reprinted with permission from Udugamasooriya et al. (2008). Copyright © 2008 American Chemical Society

beads led to the development of a two-color cell-binding assay. 250,000 compounds were screened in a one-bead one-compound fashion, collecting those beads selectively bound to VEGFR2 expressing cells using microscopy. The chemistry used to establish the library then allowed using Edman degradation of the collected beads for identification of the peptoid ligands. Consecutive dimerization of these 2 μM hits led to the development of inhibitors with an apparent dissociation constant of 30 nM. Imaging studies employing the resulting peptoid structures as targeting devices showed promising results (De Leon-Rodriguez et al. 2010).

4 Automated Carbohydrate Synthesis

Glycosylation is the most abundant posttranslational modification. Many mammalian lectins have evolved to recognize these structures orchestrating key aspects of health and disease such as development, immune response, and host-pathogen recognition. Thus, the identification and characterization of glycan-binding proteins has attracted much attention. The resulting insights into glycobiology gave rise to novel pharmaceutical targets. Such targets are C-type lectins (Geijtenbeek et al. 2000; Lasky 1992), Siglecs (O'Reilly and Paulson 2009), bacterial adhesion factors (Pieters 2007), and galectins (Liu and Rabinovich 2005; Sorme et al. 2005). Moreover, viral cell entry and release are classical examples for pharmaceutical intervention utilizing carbohydrate-based drugs. The most prominent drugs are zanamivir and oseltamivir that target influenza neuraminidase (Kim et al. 1997; von Itzstein et al. 1993). Interestingly, more than 40 viruses include sialic acid recognition as part of their life cycle (Angata and Varki 2002), and many resemble suitable targets for intervention, e.g., adenovirus causing epidemic keratoconjunctivitis (Nilsson et al. 2011). Recent progress in carbohydrate chemistry has fostered many chemical biology approaches in glycobiology (Lepenies et al. 2010), leading to the identification and characterization of novel carbohydrate binding proteins (Ernst and Magnani 2009). Hence, carbohydrates as scaffolds for medicinal chemistry can be considered as "privileged structures." Still, carbohydrate-based vaccines are the most successful examples that arose from the progress in carbohydrate chemistry (Seeberger and Werz 2005), while targeting glycan-binding proteins is still rather limited. Few drugs are available on the market or are in development (Ernst and Magnani 2009; Seeberger and Rademacher 2014). The reluctance toward using carbohydrates as a scaffold for drug design originates from a pharmacokinetic standpoint as they are readily excreted and no oral bioavailability is provided due to their high hydrophilicity. Furthermore, mammalian lectins naturally have low affinities for their substrates (Gagneux and Varki 1999; Varki 2006). Many lectins have shallow binding sites, classifying these proteins as undruggable (Hopkins and Groom 2002). However, recent examples show that advances in drug design provide grounds for reestablishing lectins as drug targets (Aretz et al. 2014; Shelke et al. 2010).

For many years, progress in the field of glycomimetics was hampered by the poor synthetic accessibility of carbohydrates compared to other biopolymers. But since the advent of automated carbohydrate synthesis, these structures have come within reach (Plante et al. 2001). Limitations such as low availability of these structures in sufficient quantities and reduced synthetic effort have been overcome (Seeberger and Werz 2005). During automated carbohydrate synthesis, the reducing end sugar is coupled to a solid support, while mono- and disaccharide building blocks are employed in coupling/decoupling cycles using sophisticated protecting group chemistry (Fig. 4). Here, similar to peptide and oligonucleotide chemistry, UV-active protecting groups are used to monitor the progress of the reaction. For the automated synthesis of carbohydrates, phosphates, thiols, and trichloroacetimidates have been proven to be useful leaving groups (Plante et al. 1999; Routenberg Love and Seeberger 2004; Seeberger 2008).

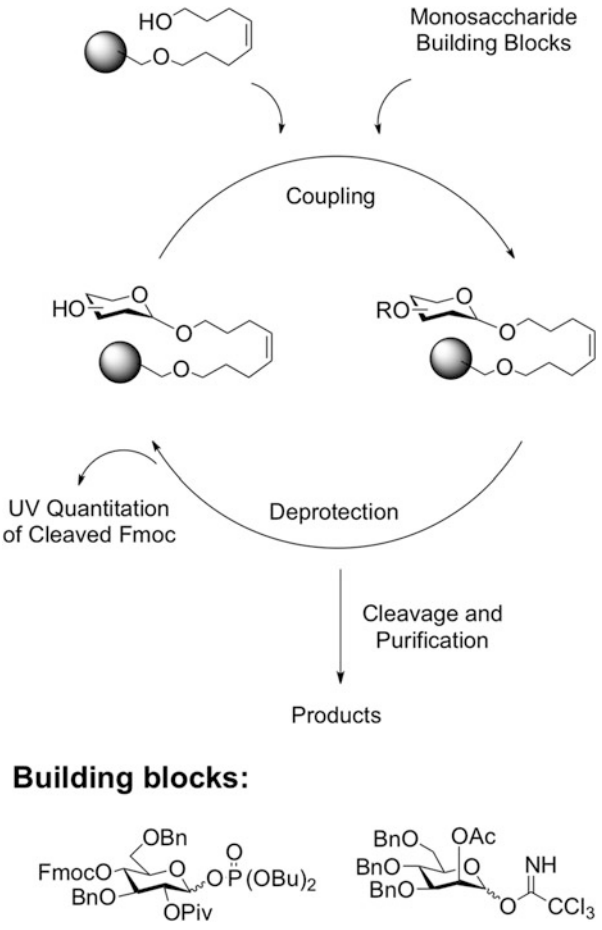

Fig. 4 Automated carbohydrate synthesis. A coupling cycle for automated carbohydrate synthesis (*top*) exemplifies the key steps. Two representative building blocks are shown (*bottom*) (Seeberger 2008)

For vaccines and carbohydrate-based diagnostics, automation of carbohydrate synthesis has large potential (Lepenies et al. 2010; Seeberger and Werz 2005). Additional applications using choosing glycans as starting scaffolds may prove to be superior to any other biopolymer due to their intrinsic building block diversity. Moreover, glycans are branched and thereby enable higher structural complexity compared to other linear biopolymers (Laine 1994). They are stereochemically rich, and some structures resemble rigid scaffolds that have up to five hydroxyl groups for substitution.

Besides their function as a starting scaffold in combinatorial chemistry, carbohydrates can also be used to access pharmacologically highly useful structures through chemical transformation. Using a Pd-catalyzed domino reaction under microwave irradiation, Werz and coworkers prepared a library of chromans and isochromans from bromoglycal as a starting material (Leibeling et al. 2010). This study opens doors for the generation of highly functionalized scaffolds for libraries from carbohydrates in high-throughput synthesis.

5 Biology-Oriented Synthesis

Why are peptides and carbohydrates successful starting scaffolds in the quest for new biologically active molecules? Waldmann and coworkers addressed this question in more general terms (Wetzel et al. 2011), extending it to all natural products by asking: Which areas of the chemical space are enriched with potentially active compounds? Structural biology efforts from many research laboratories and structural genomics initiatives fostered fundamental insights into protein structure and function, highlighting that only a limited number of protein folds exist (Redfern et al. 2008; Worth et al. 2009). Estimates for the number of polypeptide folds range around 1,000 (Koonin et al. 2002). Therefore, the legitimate assumption is made that only a certain fraction of the chemical space is able to interact with proteins in general. Evolution might already have picked the most promising small-molecule scaffolds, namely, natural products. Hence, inspiration from the coevolution of natural products and the machinery that makes them led to the "biology-inspired synthesis" (BIOS) approach (Wetzel et al. 2011). Coming from this fundamental insight, BIOS can address the central question of high-throughput synthesis, namely, selecting the correct molecular skeleton. Diversity is then introduced by chemical transformation of the scaffold with a broad variety of substituents. Hence, major initial investment in exploratory organic synthesis of natural products is highly important for this approach (Young 2010). Therefore, the key idea is that a few naturally occurring protein folds, decorated with amino acid side chains, interact with a few naturally occurring natural product scaffolds, decorated with a diverse set of appendances. It became evident that nature is not the only source of inspiration for scaffolds potentially harboring biological activity. Existing drugs were taken into account, emphasizing the key criterion for selection being biological relevance not biological existence (Wetzel et al. 2011).

In contrast to previously described approaches, in particular peptides and peptoids, BIOS does explicitly not result in extremely large libraries. It is rather the rational selection of a few "privileged scaffolds" combined with sophisticated diversification schemes enables screening for lead structures. As many natural products are not available for synthesis, in particular for high-throughput synthesis, natural product-like structures are employed. While providing close structural similarity to natural products, these molecules are more accessible to de novo synthesis. Consequently, biologically actives are prepared from focused libraries of core skeletons from solid-phase asymmetric synthesis readily diversified by chemical transformations. The upfront investment into sophisticated chemistry is higher but is rewarded with high hit rates of synthetically approachable skeletons.

In a solid-phase total synthesis of jasplakinolide and chondramide C (Fig. 5), Waldmann and coworkers presented a series of simplified biologically active

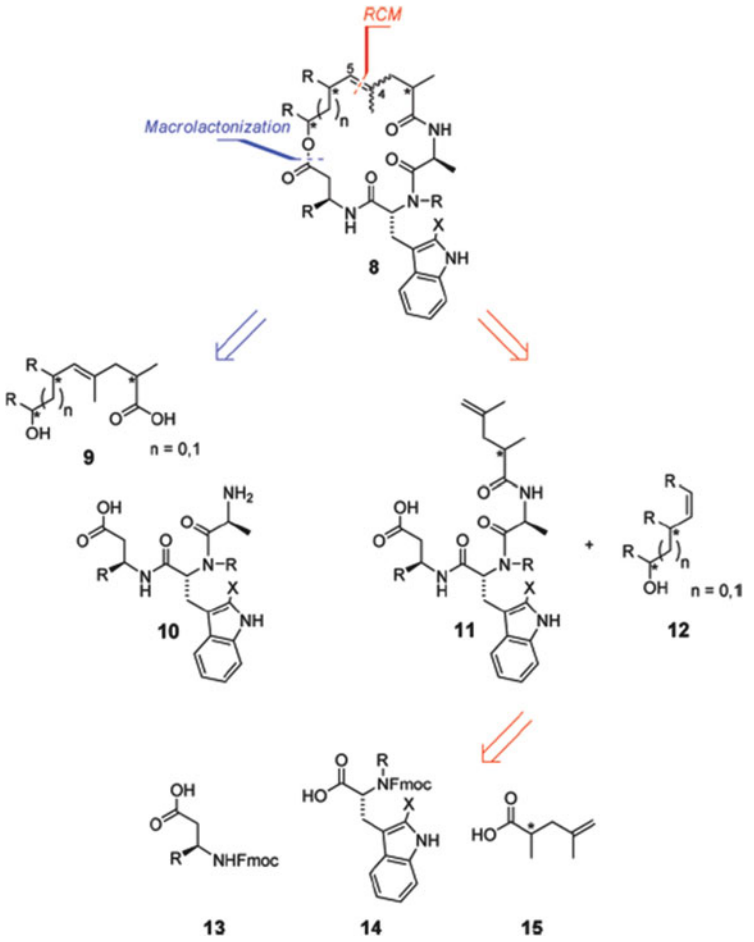

Fig. 5 Retrosynthesis of chondramide C and jasplakinolide analogs. Reprinted with permission from Tannert et al. (2010). Copyright © 2010 American Chemical Society

compounds for selective perturbation of actin-mediated cellular processes (Tannert et al. 2010). Forming the macrocycle in a ruthenium-catalyzed ring-closing metathesis from a linear peptide-based chain, a small focused library was prepared. It was found that the stereochemical configuration of the polyketide region significantly influenced the enantioselectivity. A cell-based assay was then implemented to identify chemical modulators of the actin cytoskeleton. This is a good example for a high upfront investment into sophisticated chemistry that finally led to high hit rates and novel compounds.

6 Diversity-Oriented Synthesis

The strategic design of a structurally diverse library with minimal cost has many levels of complexity, and previously described approaches have favored a diversification of the appendages, while limited scaffold diversity was incorporated. In contrast, diversity oriented synthesis (DOS) aims to diversify at three levels: appendages, stereochemistry, and the starting scaffold. Inspiration from natural products in DOS comes from the awareness that molecular complexity correlates well with biological activity (Burke and Schreiber 2004). Here, complexity of a molecule is defined as the number and variety of rigidifying elements. In this respect, natural products or those scaffolds inspired by them are more spherical and therefore are able to span their extremities into the pockets of a potential target untouched by flat scaffolds. The scaffold then determines the molecular topology of the members of a screening library. Diversifying the scaffold can then consequently maximized spatial presentation of pharmacophores (Sauer and Schwarz 2003). At the same time, more room is provided, physically and synthetically, to optimize these structures compared flat skeletons from traditional medicinal and combinatorial chemistry.

The available protein folds and consequently the fraction of all available molecules being able to interact with this limited set of general protein scaffolds resulted in BIOS. Picking up the idea of protein folding from a rather different perspective, Schreiber and coworkers came up with another solution in their quest for structurally diverse scaffolds (Burke and Schreiber 2004). The lesson they learned from protein architecture was that folding of a polypeptide chain is determined by pre-encoded information that leads to the adoption of a specific fold. Translating this principle back into organic synthesis, so-called σ-elements in the precursor building blocks are employed, encoding for the later scaffold formation. In a clever combination with other preforming σ-elements, the library can then be directed toward the formation of distinct scaffolds. In another extreme, the same σ-elements are employed, but variation of the reaction conditions drives the generation of diverse scaffolds (Burke and Schreiber 2004). As a general principle, folding-type DOS uses ring-closing metathesis of simple building blocks to explore complex molecular scaffolds. As these approaches result from fundamental insight into diastereoselectivity of the reagents and their transformations, sophisticated organic synthesis is the key to a successful DOS route.

The final level of complexity comes with appendage diversification. This can either be employed on a diverse set of scaffolds (vide supra) or simply serve as diversification of a single scaffold as found in lead optimization procedures. In the latter case, common reagent-based routes are followed to explore the chemical

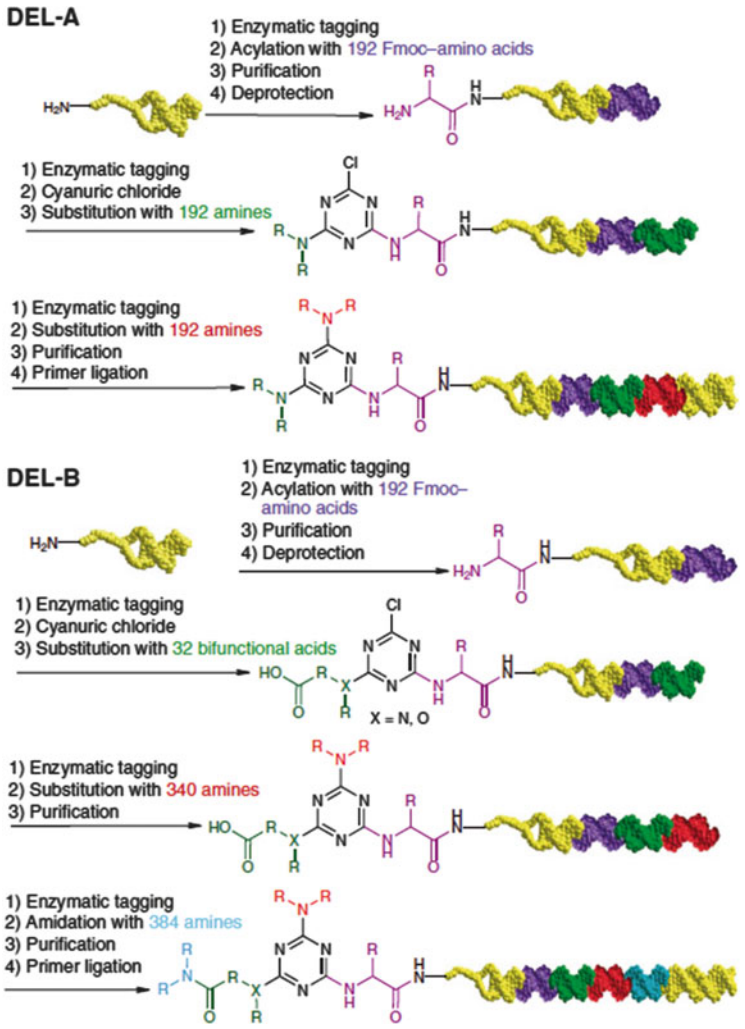

Fig. 6 Target-oriented high-throughput synthesis of two DNA-encoded small-molecule libraries DEL-A and DEL-B. A DNA duplex headpiece is conjugated with a 4,7,10,13-tetraoxapentadecanoic acid (AOP) as a spacer, and following deprotection, the resulting amine allows further diversification. This diversification is then encoded in the DNA utilizing the non-linked two-base 3′ overhang as attachment site for a 7-base variable stretch. DEL-B incorporates an additional p38 kinase pharmacophore, the 3-amino-4-methyl-*N*-methoxybenzamide (AMMB) fragment. Adapted by permission from Clark et al. (2009). Copyright © 2009 Macmillan Publishers Ltd: Nature Chemical Biology

space transforming densely functionalized compounds. Diversification can then be implemented either by allowing a limited repertoire of reactions to decorate a pluripotent scaffold or by altering reaction conditions on a simple skeleton (Burke and Schreiber 2004). Finally, any route taken during DOS results in a compound selection being smaller in size compared to traditional libraries, but structurally more complex in particular with respect to higher stereochemical variability. An attractive approach by Tan and coworkers using DOS in synthesis of a library of macrocycles via oxidative ring expansion highlights many aspects of this chapter (Kopp et al. 2012).

7 Target-Oriented Synthesis and Lead Optimization

While most high-throughput synthesis approaches presented thus far were predominantly focused on driving the library to maximal diversity, target-oriented synthesis (TOS) narrows down the diversity with respect to a defined target protein structure (Schreiber 2000). Thereby, TOS resembles more traditional synthesis efforts in pharmaceutical industry. There are many excellent examples that utilize a targeted approach exploring the binding site of a defined target, but at the same time implementing sophisticated high-throughput chemistry. One such example comes from Morgan and coworkers and utilizes an affinity-based screening technique. A library of 800-million DNA-encoded small molecules on the basis of a previously explored pharmacophore of Aurora A kinase and p38 MAPK was screened (Clark et al. 2009). This is the first report of DNA-encoded libraries (DEL) being utilized for identification of enzyme inhibitors (Fig. 6).

8 Conclusion

There is an emerging discrepancy between the augmented identification of potential drug targets and the lack of novel chemical entities being successful in drug design campaigns. High-throughput chemistry is a major motor to fill the gap by opening new doors to innovative libraries and lead optimization schemes. In that, a paradigm change has occurred, setting up new measures for the success of such a high-throughput synthesis. Sheer numbers of compounds as a sole measure for potential success are not applicable anymore. More than ever, innovative organic synthesis is the key feature that opens new avenues in drug discovery. Many approaches have diverged from the original thought of combinatorial chemistry or entered the field from various facets of chemistry, and none has proven to give the simple answer the field is looking for. Therefore, current strategies have to prove useful or will remain mere academic exercises.

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Sources for Leads: Natural Products and Libraries

Eric F. van Herwerden and Roderich D. Süssmuth

Contents

Abstract

Natural products have traditionally been a major source of leads in the drug discovery process. However, the development of high-throughput screening led to an increased interest in synthetic methods that enabled the rapid construction of large libraries of molecules. This resulted in the termination or downscaling of many natural product research programs, but the chemical libraries did not necessarily produce a larger amount of drug leads. On one hand, this chapter explores the current state of natural product research within the drug discovery process. On the other hand it evaluates the efforts made to increase the amount of

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leads generated from chemical libraries and considers what role natural products could play here.

Keywords
Diversity-oriented synthesis • Drug discovery • Fragment-based drug discovery • Natural products • Privileged structures • Unexplored natural product sources

1 Introduction

For thousands of years, man has found sources in nature to treat his ailments. With the emergence of early civilizations and the development of written language, soon the first efforts were made to record medical practices. Of course, many of the described remedies were heavily influenced by religious practices or superstition and were ineffective, but some are still used today, such as morphine (**1**) as analgesic from the *Papaver somniferum* plant (first described by the Mesopotamians) and artemisinin (**2**) as anti-malaria agent from the *Artemisia annua* plant (first described in Chinese traditional medicine) (Fig. 1).

The emergence of organic chemistry in the nineteenth century enabled the synthesis, extraction, and modification of natural products to improve their usefulness as medicines. Added to that, increased interest in microbiology led to the realization that many diseases are caused by pathogens.

While the first compounds developed to combat these pathogens were synthetic compounds (e.g., the sulphonamides by G. Domagk), the serendipitous discovery of penicillin from *Penicillium chrysogenum* by Fleming in 1928 gave a large boost to natural product research. Not long after the successful introduction of penicillin, many more classes of antibiotics from natural sources were discovered as well as other compounds with useful bioactivities such as immunosuppression (cyclosporin A), cytotoxicity (taxol), and cholesterol lowering (lovastatin). These compounds had a major impact on healthcare and therefore contributed to a considerable increase in life expectancy and quality of life during the second half of the twentieth century in the western world. Thanks to these success stories, interest in natural product research was at an all-time high.

However, the development of high-throughput screening (HTS), combinatorial chemistry, and fragment-based screening led to a decreased interest in natural

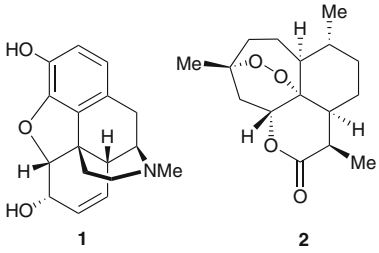

Fig. 1 Structures of morphine (**1**) and artemisinin (**2**)

product research within the industry. HTS and fragment-based screening allowed for the quick screening of entire libraries of molecules against multiple targets, and combinatorial chemistry allowed for the rapid construction of vast molecule libraries. Because natural product extracts are complex mixtures of compounds, HTS screening often generates false positives due to the presence of highly reactive components (Rishton 1997) or false negatives due to low concentrations of active components. Assay-guided fractionation of extracts and structure elucidation of extract components is therefore often required to increase hits in HTS, which is very time-consuming.

As the pharmaceutical industry shifted their focus from fields like anti-infectives towards more profitable therapeutic areas such as heart disease and diabetes, many pharmaceutical companies either discontinued or scaled down their natural product research divisions and chemical libraries became more important.

While combinatorial chemistry and biotechnological advancements promised to revolutionize the pharmaceutical industry, they have not necessarily led to an increase in productivity; while the drug discovery and development costs have exponentially increased, the amount of approved drugs has been steadily decreasing (Scannell et al. 2012). While numerous possible causes could be identified, one major cause is related to how a screening library is constructed.

This chapter assesses the relevance of natural products in drug discovery today by describing how established and (relatively) unexplored sources for natural products either resulted in drugs on the market or could be promising drug leads. Furthermore, this chapter describes how industry and academia try to improve the filling of the drug development pipeline with the aid of new approaches to chemical library construction.

2 Natural Products

Natural products with useful medical properties are commonly termed as secondary metabolites, i.e., not essential for survival. It was originally thought that these metabolites were waste products that serve no specific purpose. However, later it was hypothesized that there must be a specific evolutionary advantage for the organism to produce these metabolites as their synthesis requires a lot of energy. It was then realized that many known bioactive compounds are in fact secondary metabolites which are involved in various tasks such as defense against other organisms and repair of internal damage (Williams et al. 1989).

Natural products can be isolated from a wide range of sources. Species like plants, fungi, and bacteria yield the most natural products, possibly because their lack of mobility forces these species to rely on chemical warfare to defend itself against predators or other species competing for space and nutrients.

While many natural products originate from more traditional sources such as plants as well as bacterial and fungal strains, more recently marine life has also proven to be good source for a wide variety of natural products. These all have their advantages and disadvantages. Plants are often easy to cultivate, but production of

the desired natural product is often low and the extraction process can be time-consuming and complicated. Bacteria and fungi on the other hand are more adaptable for industrial production of natural products, but either proper cultivation protocols for the strains must be known or the biosynthesis of the compound of interest must be understood to allow for metabolic engineering or heterologous expression into strains which are easier to cultivate and manage. For marine life, their submersed environment allows for better compatibility with bioreactors and fermenters, but marine life sources have the same disadvantages as bacteria and fungi.

2.1 Recent Examples of Natural Products in Drug Discovery

As stated earlier, many big companies in the pharmaceutical industry have either downscaled or shut down their natural product research divisions, but what does this mean for the role that natural products play in current drug research? In an ambitious attempt to map out the effect natural product research has had on drug development, Newman and Cragg have written a series of extensive reviews where they investigate what percentage of all approved drugs from 1981 to 2010 are either natural products, natural product derivatives, or synthetic mimics of natural products (Newman and Cragg 2007, 2012). Although their research shows that the percentage of drugs with natural product origins to be approved indeed undergoes a steep decline from 40% in 1994 to 12% in 1997, they also show that in the following years, this percentage steadily increases again, varying between 24 and 45%, with a peak at 50% in 2010.

Because much has already been written on well-known natural product drugs like Taxol and the β-lactams, we will only focus on more recent examples. For an extensive overview, the reader is directed to the books, e.g., "Molecules that changed the World" (Nicolaou and Montagnon 2008).

2.1.1 Plants

Ingenol Mebutate (Picato®)

Ingenol mebutate (**3**, Fig. 2) is a new small-molecule (430.5 Da) diterpene ester which has been approved in 2012 for the treatment of actinic keratosis. Ingenol mebutate is an unmodified natural product isolated from the plant species *Euphorbia peplus*. This plant has a long history of being used in traditional herbal remedies to a variety of ailments, including skin cancers and keratosis (Weedon and Chick 1976). Component extraction research of this plant resulted in the identification of a number of diterpene esters which all were capable of producing an inflammation reaction, of which ingenol mebutate was identified as the cytotoxic compound (Rizk et al. 1985). It exerts its mechanism of action by targeting mitochondria. It was demonstrated that mitochondria begin to swell after exposure to ingenol mebutate. Due to this swelling, the mitochondrial membrane potential is lost, effectively shutting down the available energy within the cell due to a depletion

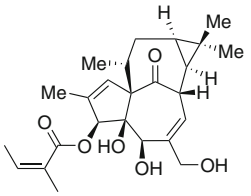

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Fig. 2 Structure of cytotoxic natural product ingenol mebutate (**3**)

of ATP. This then ultimately leads to necrosis of the cell (Ogbourne et al. 2004). Because of this mechanism, the chances of tumors developing resistance are low since most tumor drug-resistance genes expressed are apoptose-resistant genes (Ivanov et al. 2003). Besides mitochondrial swelling, ingenol mebutate also binds to protein kinase C, activating the associated signaling pathway. This activation triggers an inflammation reaction and subsequent elimination of residual tumor cells by antibodies (Kedei et al. 2004).

It is possible that in the near future, ingenol mebutate will be approved for other types of skin cancer, as the drug is also under clinical evaluation for superficial basal cell carcinoma (Siller et al. 2010). While the first total synthesis was published in 2002 (Winkler et al. 2002), a highly efficient total synthesis was published recently, which could possibly be more favorable than the current biotechnological production methods (McKerrall et al. 2014).

2.1.2 Terrestrial Fungi

Statins: Mevastatin and Lovastatin

An important and almost classical group of medication from fungal origin is the statins (Fig. 3). The statins originate from two natural compounds: mevastatin (**4**) and lovastatin (**5**). Mevastatin was isolated from *Penicillium citrinum* (Endo et al. 1976), and lovastatin was isolated from *Monascus ruber* (Endo and Monacolin 1979). Although mevastatin and lovastatin are structurally very similar, clinical development of mevastatin was discarded (Endo 2004), while clinical development of lovastatin continued and as a consequence lovastatin was the first statin to be approved. Soon thereafter, the semisynthetic derivatives simvastatin (**6**) and pravastatin (**7**) were developed and approved. Their introduction has had an enormous impact on treatment of cardiovascular diseases, and the statins are among the most-prescribed drugs worldwide. The statins exert their mechanism of action through the inhibition of hydroxyl-methylglutaryl coenzyme A (HMG-CoA) reductase. The inhibition of this enzyme prevents the conversion of HMG-CoA into mevalonic acid, thereby disrupting the mevalonate pathway which is responsible for the biosynthesis of cholesterol (Alberts 1988). As high cholesterol levels are associated with coronary heart disease (Lewington et al. 2007), the reduction of cholesterol levels significantly reduces the chances of a heart attack or stroke (Law et al. 2003).

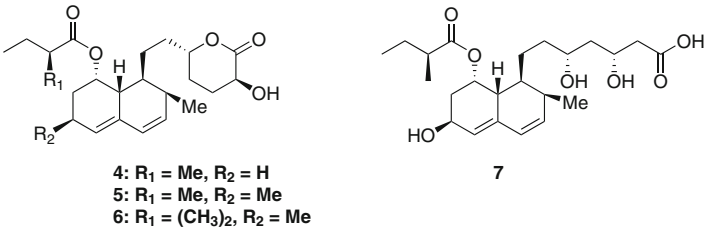

Fig. 3 Structures of natural products mevastatin (**4**) and lovastatin (**5**), and semisynthetic derivatives simvastatin (**6**) and pravastatin (**7**)

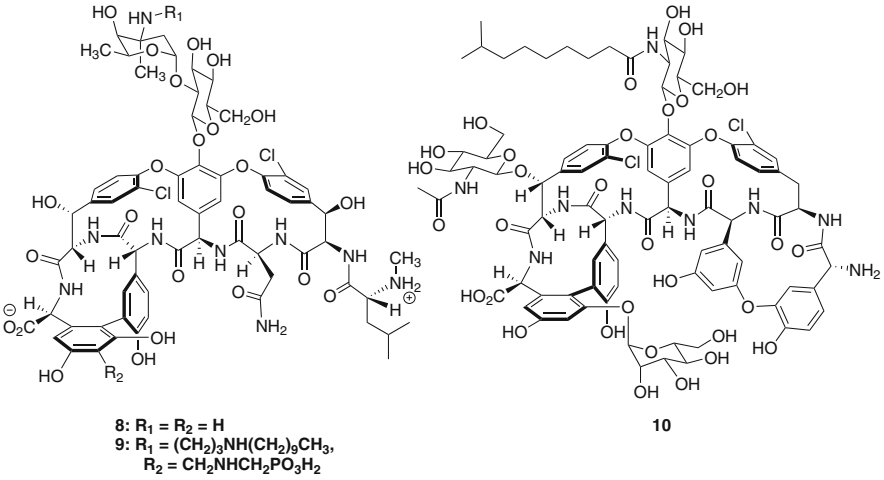

Fig. 4 Structures of the glycopeptide antibiotics vancomycin (**8**), teicoplanin (**10**), and the semisynthetic derivative telavancin (**9**)

2.1.3 Soil Bacteria

Vancomycin

Possibly one of the most important antibiotics for clinical use is vancomycin (**8**, Fig. 4). Vancomycin is a nonribosomally synthesized tricyclic glycopeptide which was originally isolated from the bacterial strain *Streptomyces orientalis* (Levine 2006). It has been approved for the treatment of various Gram-positive bacterial infections that show resistance against conventional antibiotics. Vancomycin inhibits bacterial cell wall synthesis by targeting and binding to bacterial cell membrane precursor lipid II. More specifically, it binds to the D-Ala-D-Ala moiety of the pentapeptide tail of lipid II, thereby preventing penicillin-binding proteins from cross-linking two peptidoglycans together. This in turn leads to a bactericidal cascade comparable to the β-lactams (Jordan 1961).

Although vancomycin showed high bactericidal activity, its unfavorable pharmacokinetic properties meant it could only be administered intravenously. Furthermore, early clinical trials with not sufficiently pure compounds showed serious toxicity, which is why vancomycin was almost discarded. However, with the ever-increasing bacterial resistance encountered with the early β-lactams and later even lactamase-resistant β-lactams like methicillin, vancomycin came back in the picture again. Experiments showed that bacterial resistance against vancomycin developed much slower compared to penicillin (McGuire et al. 1955). Therefore, vancomycin was further developed and approved to be used as a last-resort antibiotic only.

Although vancomycin has been used successfully as a last-resort antibiotic for many years, various strains have developed resistance mechanisms. While alternatives for vancomycin have been developed, such as the related glycolipopeptide teicoplanin (**9**), from *Actinoplanes teichomyceticus* (Somma et al. 1984), and telavancin (**10**), a recently approved semisynthetic derivative of vancomycin, various bacterial strains are also resistant to teicoplanin due to the similar mechanism of action. Telavancin was demonstrated to retain efficacy with various vancomycin-resistant strains (Krause et al. 2008), but as the MIC values of nonresistant and resistant strains increase from ng/mL to μg/mL, telavancin resistance should be expected in the near future.

Daptomycin

Daptomycin (**11**, Fig. 5) is one of the few antibiotics with a novel mechanism of action to be approved in the last few decades. Originally isolated as a fermentation product from bacterial strain *Streptomyces roseosporus* (Tally and DeBruin 2000), daptomycin is a nonribosomally synthesized cyclic lipopeptide containing a lipid tail and various D-amino acids (Mao et al. 2005). It has been approved for the treatment of various Gram-positive infections to the skin and endocarditis.

Daptomycin has a unique mechanism of action which requires the binding to calcium. Once daptomycin has complexed with calcium, it inserts itself into the

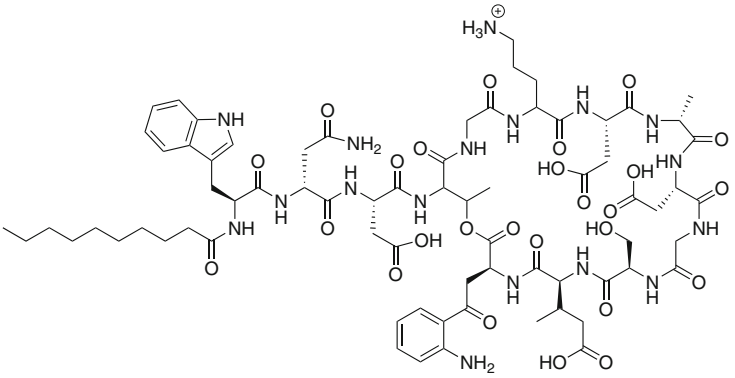

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Fig. 5 Structure of antibacterial natural product daptomycin (**11**)

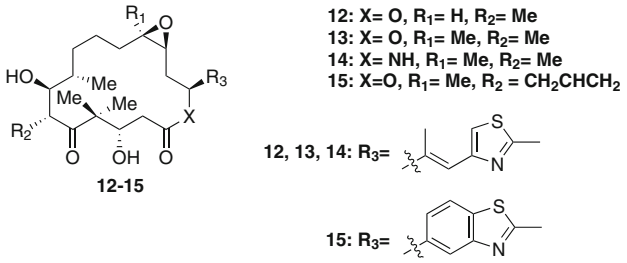

Fig. 6 Structures of cytotoxic natural products epothilone A (**12**) and B (**13**), semisynthetic derivative ixabepilone (**14**), and synthetic epothilone analogue sagopilone (**15**)

bacterial cell membrane, where it destabilizes the membrane by causing lipids to flip flop. This causes leakage and loss of membrane potential, leading to bacterial cell death (Silverman et al. 2003; Jung et al. 2004). During clinical trials, only a very small amount of patients displayed resistance towards daptomycin. For these cases, it has been shown to be the result of gene mutations which made the bacterial strains less susceptible, but not necessarily fully resistant (Arias et al. 2011). As a result of this, daptomycin will most likely retain an important role in clinics as last-resort antibiotic with a low risk of resistance for many years so come.

Epothilones

Originally isolated from the myxobacterial strain *Sorangium cellulosum* (Höfle et al. 1996), the epothilones (Fig. 6) are a group of macrolactones that were first thought to be useful antifungal compounds, until researchers at Merck showed that epothilones A and B had cytotoxic activity against multiple drug-resistant cancer cell lines (Bollag et al. 1995). This led to a fierce competition among Merck, Bristol-Myers Squibb, Schering-Plough (now merged with Merck), and Novartis, who all recognized the great potential of the epothilones as successors of the taxanes.

Although the epothilones showed great potential, it was soon discovered that their lactone ring was vulnerable to cleavage (Hunt 2009). To overcome these issues, extensive testing of semisynthetic derivatives was undertaken, which was made possible by learning from the total syntheses of epothilones A (**12**) and B (**13**) (Balog et al. 1996; Schinzer et al. 1997; Nicolaou et al. 1997). Ultimately, it was discovered that simply replacing the lactone for an amide solved the metabolic instability. This led to the development of epothilone analogue ixabepilone (**14**) by researchers at Bristol-Myers Squibb, after which approval was granted a few years ago. The development of synthetic epothilone by Bayer AG (formerly Schering AG), sagopilone (**15**), seems currently set on hold.

Carfilzomib (Kyprolis®)

Carfilzomib (**16**, Fig. 7) is a new small tetrapeptide (719.9 Da) with anticancer properties which has been approved in 2012 for the treatment of patients with relapsed and refractory multiple myeloma. It belongs to the new class of

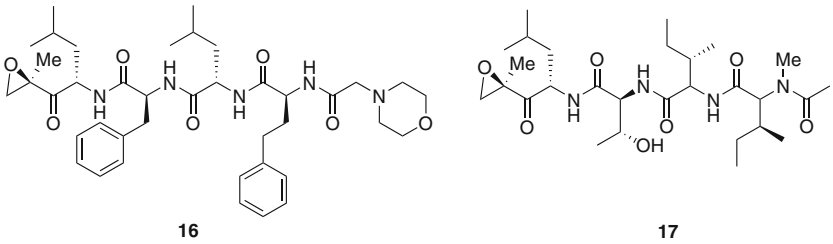

Fig. 7 Structures of the semisynthetic derivative carfilzomib (**16**) and the natural product epoxomicin (**17**)

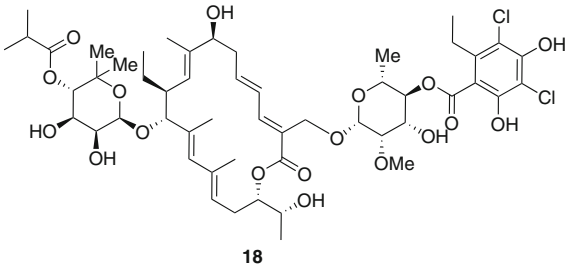

Fig. 8 Structure of the antibacterial natural product fidaxomicin (**18**)

proteasome inhibitors. Carfilzomib is based on epoxomicin (**17**), a natural product which was isolated from soil *actinomycete* No. Q996-17 and contains an unusual epoxide ring (Hanada et al. 1992). The compound's mechanism of action works through the irreversible inhibition of the proteasome, leading to apoptosis in tumor cells (Meng et al. 1999). The total synthesis was soon achieved (Sin et al. 1999), after which various analogues of epoxomicin were synthesized, creating variations on the amino acid residues, resulting in carfilzomib (Halford 2012).

Fidaxomicin (Dificid®)

Fidaxomicin (**18**, Fig. 8) is a new, first-in-class macrocyclic (1,072.1 Da) antibiotic which has been approved in 2011 for the treatment of *Clostridium difficile* bacterial infections in the gastrointestinal tract. It is one of the few unmodified natural products to be approved, and it belongs to a family of polyketide fermentation products of the soil actinomycete *Dactylosporangium aurantiacum*, known as the lipiarmycins/tiacumicins (Parenti et al. 1975). Although fidaxomicin shows high bactericidal activity against a wide range of Gram-positive bacteria (Coronelli et al. 1975), its size and polar surface area prevent it from being absorbed into the bloodstream in its current form. It was found however that naturally occurring gastrointestinal bacteria are relatively unaffected by fidaxomicin, which is why development of the drug has focused towards applications on the gastrointestinal tract.

The structure of fidaxomicin with obvious structural resemblance to other polyketides is unique in that the compound is bactericidal while the other macrolides are bacteriostatic. It also shares similarity with rifamycin in that it also inhibits bacterial RNA polymerase, be it in a different position: where rifamycin inhibits this process by binding to the nascent RNA chain, fidaxomicin binds to the DNA template which in turn prevents RNA polymerases from binding to the DNA (Venogupal and Johnson 2012). This unique mechanism could provide treatment for patients infected with methicillin-resistant and vancomycin-resistant bacterial strains.

Another thing of interest to note is, while fidaxomicin was already discovered in 1975, it was largely overlooked by the big pharmaceutical companies and was not approved until 2011 after being developed by a small company called Optimer Pharmaceuticals (Erb and Zhu 2013).

Ramoplanin

The peptide antibacterial ramoplanin (**19**, Fig. 9) (2,510.1 Da) has been approved in 2011 for the treatment of *Clostridium difficile* bacterial infections in the gastrointestinal tract. It is a fermentation product of the soil actinomycete *Actinoplanes* spp. ATCC 33076 (Pallanza et al. 1984). Ramoplanin shows high bactericidal activity against a wide range of Gram-positive bacteria, but it has the same limitations and advantages as fidaxomicin. The mechanism of action involves bacterial cell wall inhibition by binding to lipid II, but whereas vancomycin binds to the pentapeptide

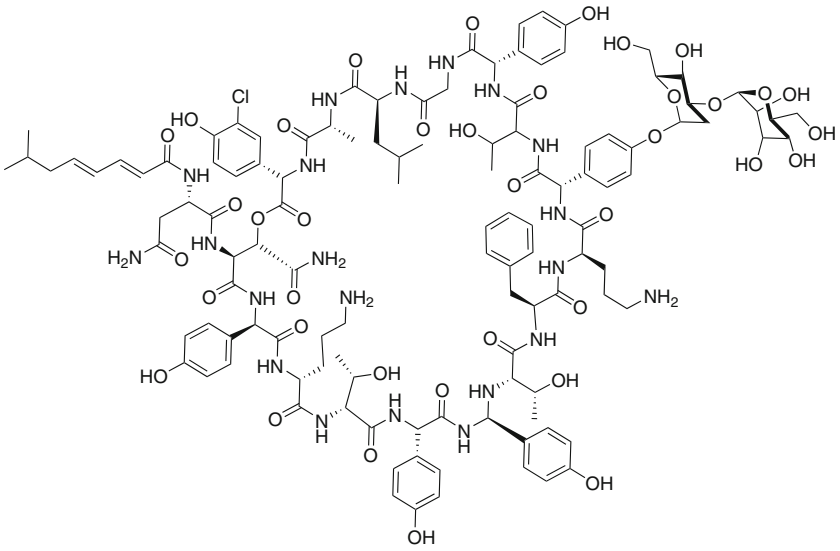

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Fig. 9 Structure of the antibacterial natural product ramoplanin (**19**)

moiety of lipid II, ramoplanin is thought to form a dimeric complex with the sugar moieties of lipid II on the outside of the bacterial cell wall (Hu et al. 2003).

2.1.4 Marine Life

Conotoxins

The conotoxins are the first group of natural products from marine life to be developed and approved as a drug. Originally isolated from marine cone snail species *Conus magus* (McIntosh et al. 1982), the conotoxins are a group of neurotoxic small peptides which contain a high amount of disulfide bridges. It was found that there are five distinct groups of conotoxins, namely, the α-, δ-, κ-, μ-, and ω-conotoxins, all targeting different receptors in the central nervous system (Olivera and Cruz 2000).

Although all of these conotoxins have interesting biological applications, only one specific ω-conotoxin, MVIIA (**20**, Fig. 10, ziconotide), a peptide 25 amino acids long which contains three disulfide bonds, has so far been developed and approved after clinical trials as an analgesic. Its total synthesis was achieved by using solid-phase peptide synthesis (Olivera et al. 1987).

Ecteinascidin-743

Ecteinascidin-743 (**21**, Fig. 11) is a marine natural product isolated from sea squirt species *Ecteinascidia turbinata* (Sigel et al. 1969). It is a potent cytotoxic agent which has been approved in 2007 for the treatment of soft tissue sarcoma. It exerts its mechanism of action through the binding to the minor groove in the DNA double helix and subsequently inducing double DNA strand breaks while preventing repairs through an unknown mechanism (Nicolaou and Montagnon 2008).

Structure elucidation of the active components of the extracts was achieved in 1990, where it was shown that ecteinascidin-743 had the highest potency (Wright et al. 1990; Rinehart et al. 1990). Soon after the structures were available, the total synthesis was achieved (Corey et al. 1996; Martinez and Corey 2000), and a semisynthetic approach that used the antibacterial natural product cyanosafractin

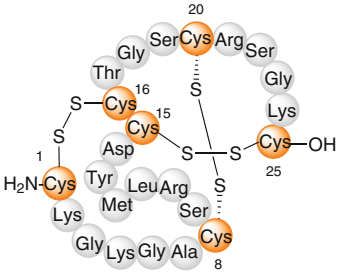


Fig. 10 Amino acid sequence of ω-conotoxin MVIIA (**20**). Disulfide bridges between Cys1–Cys16, Cys8–Cys17, and Cys15–Cys25 are drawn

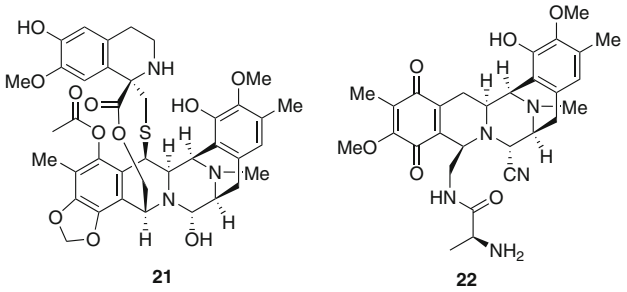


Fig. 11 Structures of cytotoxic natural product ecteinascidin-743 (**21**) and antibacterial natural product cyanosafractin B (**22**)

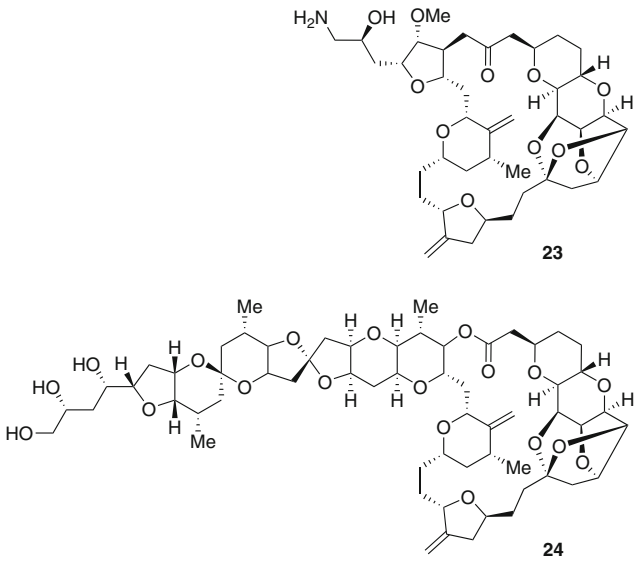


Fig. 12 Structures of cytotoxic semisynthetic derivative eribulin (**23**) and natural product halichondrin B (**24**)

B (**22**), from bacterial strain *Pseudomonas fluorescens*, as starting material provided enough material for clinical testing (Cuevas et al. 2000).

Eribulin (Halaven®)

Eribulin (**23**, Fig. 12) is a new anticancer drug which was approved in 2010 for the treatment of metastatic breast cancer. Like the vinca alkaloids and taxanes, Eribulin is a microtubule inhibitor, wherein growth of microtubules is blocked (Bai et al. 1991; Jordan et al. 2005). The development of Eribulin began with the discovery and structure elucidation of halichondrin B (**24**), a polyether macrolide

isolated from the marine sponge species *Halichondria okadai* (Hirata and Uemura 1986). The total synthesis was achieved soon thereafter (Aicher et al. 1992). Fragment-based screening and further derivatization then resulted in Eribulin (**23**, Yeung 2011).

2.2 Unexplored Sources of Natural Products

From the currently established sources for natural products, the potential of finding new natural products seems virtually limitless, when one considers that our knowledge on the plant, bacterial, and fungal kingdoms is still very limited; it is estimated that only 10% of all-known plant species have been tested for the presence of bioactive compounds (Verpoorte 1998) and about 1% of all microbes have been cultured and investigated (Hugenholtz et al. 1998). Besides these established sources, there are unexplored potential sources for natural products which have only recently received recognition from the academic community. A few examples of sources which have been shown to contain new natural products are discussed below.

2.2.1 Extremophiles, Pathogenic, and Non-cultivable Microorganisms

Extremophiles are organisms which have managed to survive in extreme environments in which the majority of life on Earth would perish. Groups of extremophiles include acidophiles and alkaliphiles living in extreme pH environments, anaerobes requiring the absence of oxygen to survive, thermophiles living in high temperature environments, and halophiles living in environments that contain high concentrations of salts.

Because extremophiles have managed to survive in these extreme environments, they have evolved unique metabolisms and survival strategies which no doubt involve the synthesis of interesting secondary metabolites. As a result of this, various natural products with a wide variety of biological activities have been discovered, such as the ribosomally synthesized lantipeptide curvopeptin from bacterial species *Thermomonospora curvata* (Krawczyk et al. 2012), cytotoxic agents variecolorquinones A and B from the halotolerant marine fungal species *Aspergillus variecolor* (Wang et al. 2007), the cytotoxic berkelic acid from an acidophilic fungal *Penicillium* species (Stierle and Stierle 2005; Stierle et al. 2006), and the antimicrobial and cytotoxic agent naphthospironone A from alkaliphilic bacterial strain *Nocardiopsis* sp. YIM DT266 (Ding et al. 2010). Not only does this demonstrate the potential of extremophiles as sources for natural products, it is also curious to note that berkelic acid and naphthospironone were in fact not isolated from naturally occurring extreme environments but from heavily polluted mining areas.

Likewise an interesting source of new structural diversity could be pathogenic microorganisms which use small molecules in host–pathogen interactions. Recent examples have been reported for the honey bee and the bacterium *Paenibacillus larvae*, the causative agent of the American Foulbrood. Bacterial spores are

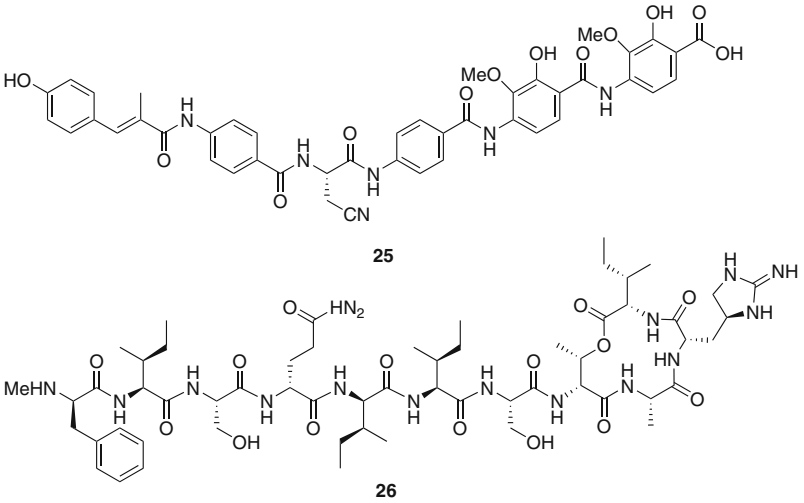


Fig. 13 Structures of antimicrobial peptides albicidin (**25**) and teixobactin (**26**)

ingested in the honeycomb by the bee larvae where they germinate. The outgrown bacterial culture breaks through the midgut lumen and kills the larvae, which ultimately could lead to a collapse of the beehive. From a cocktail of various compounds, a new structure has been identified, paenilamicin, which is a hybrid polyketide/peptide structure with interesting antibacterial and antifungal properties (Müller et al. 2014). Another example comes from plant-associated Gram-negative bacteria of the genus Xanthomonas. From these the sugarcane pathogen *Xanthomonas albilineans* causes a chlorosis in sugarcane leaves. The causative agent named albicidin (**25**, Fig. 13) has recently been identified as an unusual structure consisting almost entirely of *para*-amino benzoic acids (Cociancich et al. 2015). Albicidin inhibits bacterial gyrase and constitutes an antibacterial with strong activity against Gram-negative bacteria. The elaboration of a total synthesis (Kretz et al. 2014) and the modular structure of albicidin facilitates the synthesis of derivatives for structure activity relations and a potential development of an antibacterial.

Finally, most recently, researchers have managed to cultivate previously considered non-cultivable bacteria from soil. To achieve this, they developed and applied particular cultivation techniques (Nichols et al. 2010) which enabled the formation of small starter cultures which were able to grow further. From such a culture, the antibacterial teixobactin (**26**) has been isolated (Ling et al. 2015) which shows strong antibacterial activity against various multiresistant Gram-positive bacteria. Teixobactin targets the bacterial cell wall biosynthesis and even a multitude of passages could not generate resistance. While the above examples still await further profiling for use as drugs, these are encouraging to pursue the search of drug leads within less investigated or even untapped natural reservoirs.

2.2.2 Genomes and Genome Mining Approaches

The sequencing and publication of the human genome is often considered one of mankind's greatest scientific achievements of modern history. Scientists have also managed to sequence the genomes of a large number of fungal and bacterial strains. At first, scientists primarily used the information from these sequenced genomes for an understanding of the model organism of interest (Li et al. 2004) or to find new potential targets for the development of drugs (Miesel et al. 2003).

However, it was recently discovered that bacterial and fungal genomes contain many "silent" (cryptic) gene clusters, i.e., clusters that are not expressed, that have the potential of translating into natural products or natural product-synthesizing enzymes, provided the right activation conditions are found. Due to our increased understanding of the biosynthesis of various natural products by the enzyme groups of polyketide synthases and nonribosomal peptide synthetases (Fischbach and Walsh 2006), it is now possible to scan the genome of lesser known species for genes (Hornung et al. 2007) or gene clusters that share some homology to gene clusters from species that are known to be translated into specific natural products and/or natural product-synthesizing enzymes. Subsequently, the gene clusters of interest can then be "activated" by expression in other, better controlled systems to see if new natural products will be formed. This technique is known as genome mining.

Although genome mining is still a relatively new technique, it has already led to the discovery of a large number of new natural products. Examples of natural products discovered through genome mining include the ribosomally synthesized lantipeptides curvopeptin from bacterial species *Thermomonospora curvata*, erythreapeptin from bacterial species *Saccharopolyspora erythraea* (Völler et al. 2012) and haloduracin from bacterial species *Bacillus halodurans* (McClerren et al. 2006), nonribosomally synthesized peptide coelichelin from bacterial species *Streptomyces coelicolor* (Lautru et al. 2005), ribosomally synthesized peptides plantazolicin A and B from bacterial species *Bacillus amyloliquefaciens* FZB42 (Kalyon et al. 2011), and small molecules terrequinone A and aspyridone A from fungal species *Aspergillus nidulans* (Bok et al. 2006; Bergmann et al. 2007). By combining genome mining with metabolic engineering, a high amount of structurally diverse natural products could be obtained in the near future, possibly leading to many new drug leads.

2.2.3 Metabolic Engineering

Metabolic engineering approaches are directed towards either the overproduction of desired natural products, production of natural products in other, better producers (heterologous production), or generate entirely new natural products which would normally not be produced at all. Because overproduction and heterologous production have both more or less been established for several examples, this paragraph will focus on the generation of new natural products through metabolic engineering. A considerable number of natural products are produced by polyketide synthetases (PKS) and nonribosomal peptide synthases (NRPS) (Richard Hutchinson 2003). While this makes understanding the biosynthesis of newly discovered natural

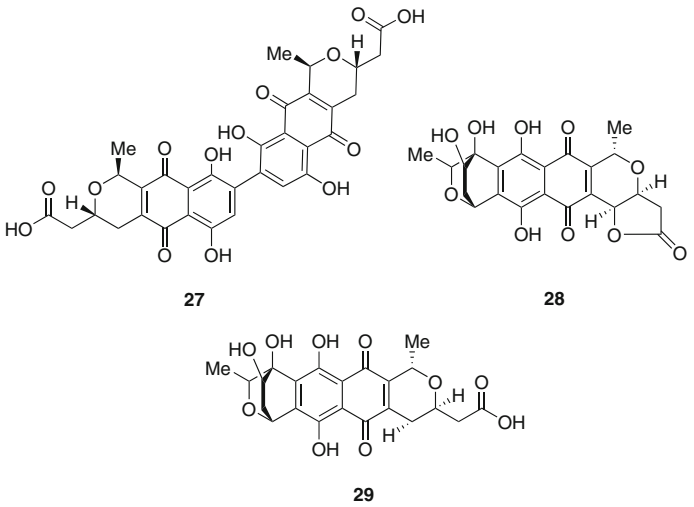


Fig. 14 Structures of antibacterial natural products actinorhodin (**27**) and granaticin (**28**), and recombinant antibiotic dihydrogranaticin (**29**)

products easier, this also means that the different types of natural products is probably limited, assuming no fundamentally new, unique biosynthetic pathways will be discovered.

For the biosynthesis of entirely new natural products, a common method used is to introduce mutations into the producing enzymes in order to either modify the produced natural product or to diversify the precursors that the enzymes will accept to incorporate into the natural product structure. The first example of this method was published in 1985, where the combination of PKS gene clusters for producing enzymes of the antibiotics actinorhodin (**27**) and granaticin (**28**) led to the production of dihydrogranaticin (**29**), which includes structural features from both antibiotics (Fig. 14) (Hopwood et al. 1985).

Another well-known example is the biosynthesis of analogues of the antibiotic erythromycin. Through mutation of the PKS, it became possible to introduce various functional groups which resembled the original precursors through feeding experiments, giving rise to a number of deglyco-erythromycin analogues (McDaniel et al. 1999, Fig. 15). In a similar way, metabolic engineering of PKS and feeding experiments has resulted in the successful production of various enniatin analogues (Feifel et al. 2007).

Metabolic engineering of NRPS has also been investigated, where it was found that rearrangement of the modules within the gene clusters could be employed to create a large diversity of peptide natural products (Mootz et al. 2002). Not long after this discovery, extensive manipulation studies of various modules from the NPRS responsible for the biosynthesis of daptomycin resulted in a range of new daptomycin analogues (Nguyen et al. 2006). Further recent examples include the

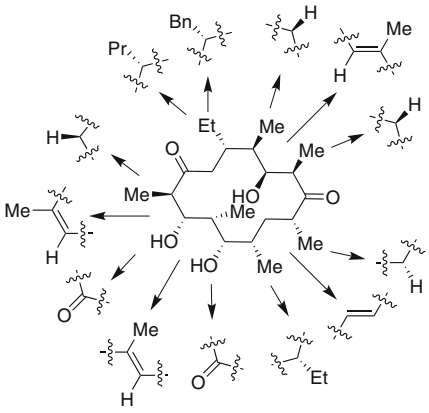


Fig. 15 Structures of various erythromycin analogues produced by metabolic engineering and feeding experiments

generation of analogues of antibacterial glycopeptide balhimycin (Butz et al. 2008), the heterologous expression and analogue generation of the two-component lantibiotic lichenicidin by both module manipulation (Caetano et al. 2011), and feeding of noncanonical amino acids (Oldach et al. 2012).

As can be seen from abovementioned examples, the biosynthesis of new natural products through metabolic engineering is rapidly gaining recognition, and it is expected that this will become an important aspect of drug discovery in the future.

3 Chemical Libraries and Privileged Structures

3.1 Combinatorial Chemistry

As stated before, the development of combinatorial chemistry allowed for the rapid synthesis of large amounts of compounds. This technique originated from the field of peptide chemistry; the introduction of a solid support to attach an amino acid to and build a peptide without intermediate purifications needed (Fig. 16, Merrifield 1963) allowed for the construction of the first combinatorial peptide libraries (Jung and Beck-Sickinger 1992). This was then adapted in the late 1980s to early 1990s to attach small core structure molecules to a solid support and subsequently modifying these (Fig. 17).

This led to the synthesis of large combinatorial libraries consisting of enormous amount of compounds which were submitted to random screening in HTS. While these early libraries achieved some degree of success in producing leads (Golebiowski et al. 2001), their hit rates were drastically low. For example, a large combinatorial library construction and HTS campaign to identify new

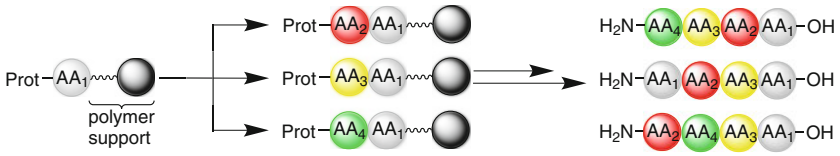


Fig. 16 Construction of a small combinatorial peptide library using a solid support. The first amino acid is immobilized onto a polymer which is insoluble in the reaction medium, allowing reagents to be washed away after every coupling. Protection group removal, amino acid couplings and subsequent global deprotection, and cleavage from the solid support afford the peptides

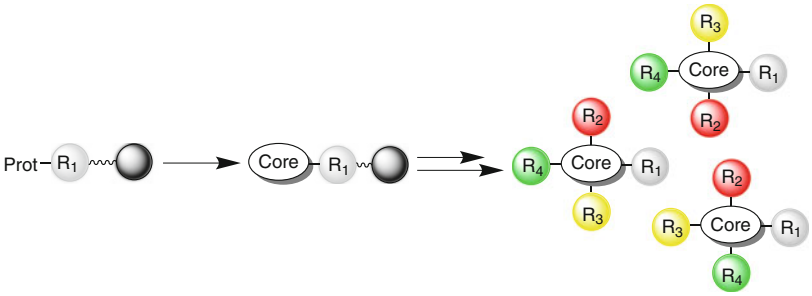


Fig. 17 Construction of a small combinatorial library using a solid support. After building up a core structure, interchangeable introduction of fragments or functional groups and cleavage from the solid support affords the compounds. Alternatively, fragments and functional groups can also be coupled directly without a core structure

antibacterial leads were very disappointing (Payne et al. 2007). To identify the causes of these low hit rates from HTS of combinatorial libraries, an extensive comparison of the physiochemical properties of the compounds within these combinatorial libraries with known natural products and drugs already on the market was undertaken (Feher and Schmidt 2003). This comparison showed that the compounds in the combinatorial libraries had lower structural diversity and complexity (i.e., less stereocenters and less ring structures), a different distribution of heteroatoms, higher hydrophobicity, and more flexibility (i.e., more rotatable bonds). These factors all result in a decrease of favorable physiochemical properties, and thus such libraries are unlikely to yield drug-like compounds.

As a consequence, the synthesis of libraries with a focus on quantity fell out of favor, and smaller libraries which contained compounds with better drug-like properties were constructed. To increase the drug-like or natural product-like properties of these libraries, multiple approaches such as fragment-based drug discovery (FBDD) and diversity-oriented synthesis (DOS) have been developed. These approaches frequently rely on the use of so-called privileged structures, which will be discussed in the following.

3.2 Fragment-Based Drug Discovery (FDBB)

FDBB is a technique where highly water-soluble fragments (molecules <300 Da) are screened for interaction with the target, after which structural information of how the fragments bind to the target guide the subsequent fragment optimization and development towards a drug lead.

FBDD arose from the idea to first identify smaller fragments that bind to the target and to develop these fragments into drug leads (Jencks 1981). The smaller size of the fragments allows for a more efficient interaction with binding pockets within the target, even though the binding affinities of the fragments are much lower (typically 0.1–10 mM as opposed to μM/nM affinities screened for in HTS). Furthermore, the amount of candidate molecules of this size is smaller, allowing for a larger structural diversity to be explored with a smaller library size.

The various approaches to lead development through FBDD are illustrated by a hypothetical example in Fig. 18. Once various binding fragments are identified and their binding interactions are understood through analytical methods such as NMR spectroscopy, MS, or X-ray crystallography, these fragments can either be recombined through merging or fusion to fully take advantage of each fragment's binding interactions. Alternatively the fragments can be expanded in an attempt to fill up the binding pocket (Scott et al. 2012).

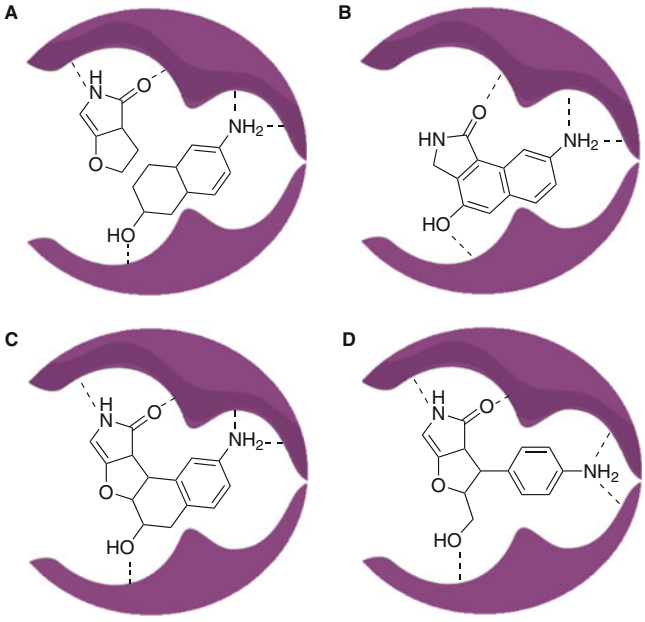


Fig. 18 The principle of fragment-based drug discovery. Various small hydrophilic fragments are screened for receptor interactions (**a**). Binding fragments attached to different sites of the binding pockets are either merged (**b**), linked (**c**), or one of the fragments could be optimized/grown (**d**) in an attempt to fill up the binding pocket (Scott et al. 2012)

Besides these three commonly used strategies, another FBDD strategy called in situ fragment assembly has rapidly gained recognition in recent years. The target is presented with various fragments which contain a bio-orthogonal functional group (i.e., a group that cannot directly react with the target) and then let the target "choose" which fragments fit best in its binding pocket. The bound fragments are then ligated in situ and the nonbinding fragments washed away (Fig. 19). While various reactions have been employed to ligate bound fragments (Mamidyala and Finn 2010), one of the most efficient and popular reactions is the copper-catalyzed azide-alkyne "click" reaction, which was employed by the group of Sharpless to assemble an acetylcholinesterase inhibitor with femtomolar inhibition concentrations (Lewis et al. 2002).

Analogous to Lipinski's "rule of five" for drug-like compounds (Lipinski et al. 1997), a "rule of three" was proposed for favorable fragment properties, which are MW $\leq$ 300 Da., number of hydrogen bond donors $\leq$3, number of hydrogen bond acceptors $\leq$3, and a ClogP value of $\leq$3 (Congreve et al. 2003). While there is still some debate on whether or not these rules hold up, they have proven to be valuable guidelines in the design of fragments (Jhoti et al. 2013).

As FBDD has been embraced by both industry and academics over the last two decades, the approach has started showing its merits in this decade. In 2011, the oncogenic agent Vemurafenib (**30**), a B-Raf kinase inhibitor which was discovered through FBDD (Fig. 20), was approved by the FDA, and multiple FBDD-based drugs are currently going through phase II/III clinical trials (Baker 2013).

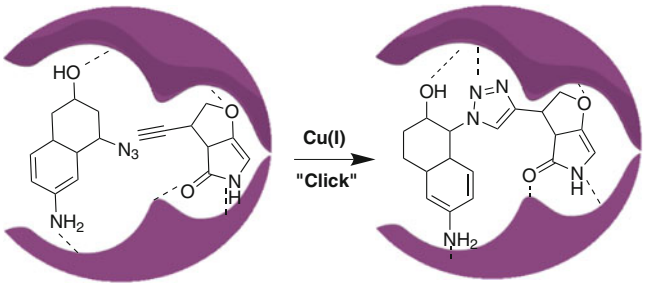


Fig. 19 In situ fragment assembly via copper-mediated "click" chemistry

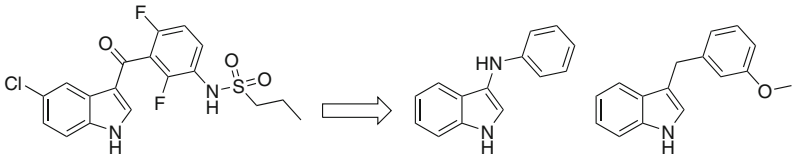


Fig. 20 Structure of Vemurafenib (**30**), which was discovered by fragment merging of indoles **31** and **32**, followed by lead optimization (Tsai et al. 2011)

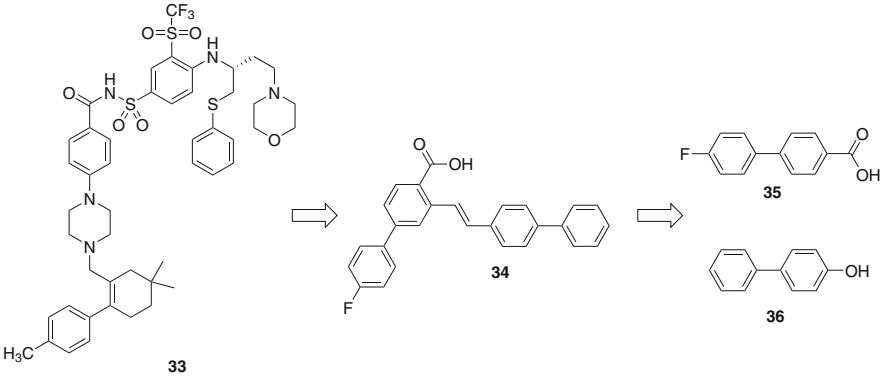


Fig. 21 FBDD approach which led to the discovery of Navitoclax (**33**). **33** was the result of optimization of tetra-aryl **34**, which in turn was the result of merging fragments **35** and **36** (Petros et al. 2006; Park et al. 2008)

Furthermore, the attention FBDD has received from academia has also led to FBDD being applied to chase after targets that were originally thought to be too difficult and risky to be approached as well as targets in less-profitable areas (Scott et al. 2012). One interesting example is the oncogenic agent Navitoclax (**33**, Fig. 21), a B cell lymphoma 2 protein inhibitor, which is currently in clinical trials. This compound in fact inhibits a protein–protein interaction, which was originally thought to be incompatible with FBDD due to the small size of the fragments.

3.3 Diversity-Oriented Synthesis (DOS)

Like FBDD, DOS was developed in response to the poor performance of early combinatorial libraries, but where FDBB aims to synthesize a small library with high structural diversity focused towards a specific target, DOS aims to attain a library with high structural diversity and molecular complexity through a series of stereospecific reactions that can be carried out in parallel (Schreiber 2000). The philosophy behind this was that small fragments, while diverse, would lack the complexity (i.e., 3-dimensional features) required for optimal target binding, while the complexity generated by DOS would yield more natural product-like compounds and thus would lead to better target binders (Hajduk et al. 2011).

Because the focus of DOS lies in generating as much diversity and complexity per reaction as possible, retrosynthetic analysis has been replaced by a similar concept called forward-synthetic analysis. Where retrosynthetic analysis starts off with a complex target and subsequently simplifies the synthons required to attain the target in every step, forward-synthetic analysis starts simple and then aims to generate as much complexity as possible within the course of typically three to five reaction steps. The challenge here then lies in using reaction procedures where the

product of a certain reaction can then be used as substrate for the next complexity-generating reaction as is illustrated in Fig. 22 (Burke and Schreiber 2004).

With respect to the rapid generation of diversity, the use of multicomponent reactions such as the Ugi reaction (Ugi 1997) have proven very useful in constructing varied scaffolds, as illustrated by the group of Yang, who synthesized two distinct isoquinoline scaffolds **38** and **39** using an Ugi reaction followed by a Heck intramolecular cross coupling (Fig. 23).

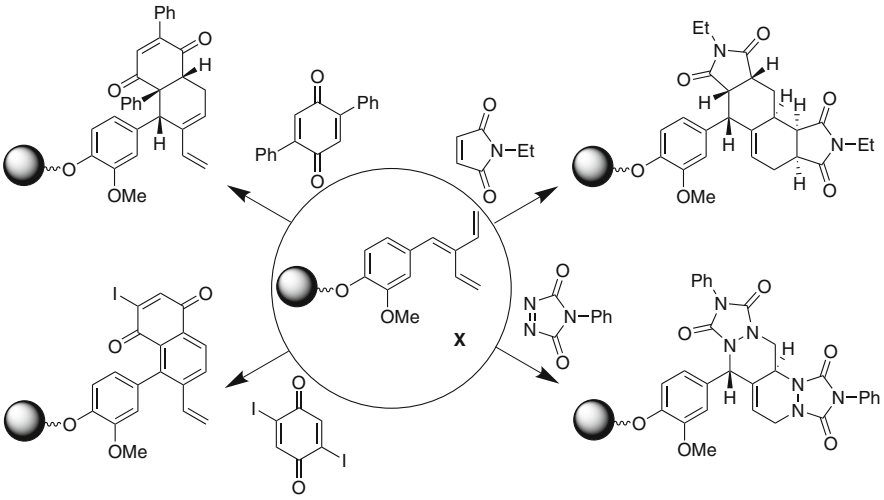


Fig. 22 DOS approach to generate various complex scaffolds from trienophenol **37** using similar reagents and similar reaction types (Kwon et al. 2002)

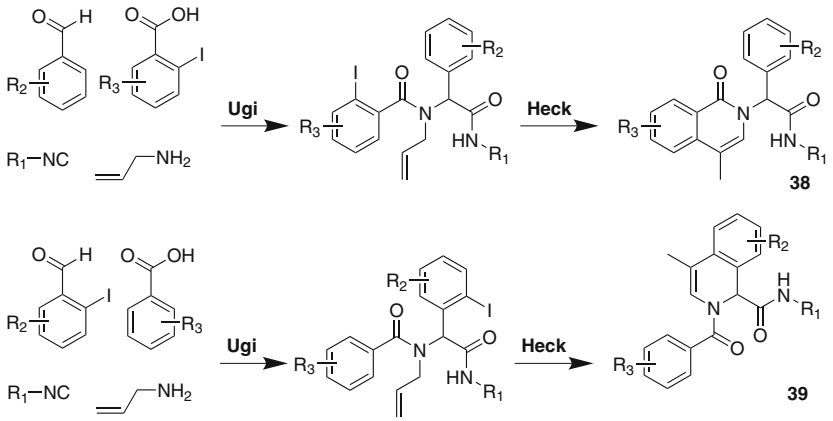
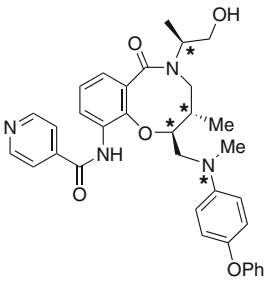
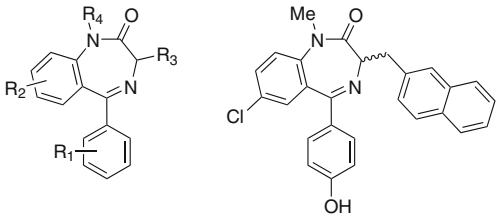


Fig. 23 Ugi multicomponent reaction between an amine, cyanide, aldehyde, and carboxylic acid to yield the substituted amino acids, which in turn are cyclized using a Heck palladium-mediated cross coupling (Xiang et al. 2004)



40

Fig. 24 Trypanocidal lead ML341 **40** resulting from an extensive DOS library of ca. 100,000 compounds. Variations in stereochemistry and attached substituents that have been screened are indicated with *asterisk*. No variation in the skeletal structure was reported (Dandapani et al. 2014)



41 42

Fig. 25 The benzodiazepine privileged scaffold (**41**) with four possible substituent sites and optimized CKK A antagonist Bz-423 (**42**)

While DOS has not been implemented to the same extent as FBDD has in industry and academia, it has still yielded a number of drug leads in the antiparasitic area, such as malaria (Dandapani et al. 2012) and Chagas disease, where an extensive DOS campaign resulted in the discovery of trypanocidal compound ML341 (**40**, Fig. 24)

3.4 Privileged Structures

Regardless if FBDD or DOS is chosen to construct a chemical library, the question is what to choose as starting point to build the library around to ensure biological relevance. One possible answer is the use of so-called privileged structures as scaffolds. Privileged structures are defined as fragments which seem to be recurring motives in nature and known drugs which have the ability to bind to one or multiple targets. The term was originally coined by the group of Evans, who discovered that the benzodiazepine scaffold (**41**, Fig. 25) did not only target the GABA neuroreceptor but in fact targets a wide array of receptors, such as cholecystokinin

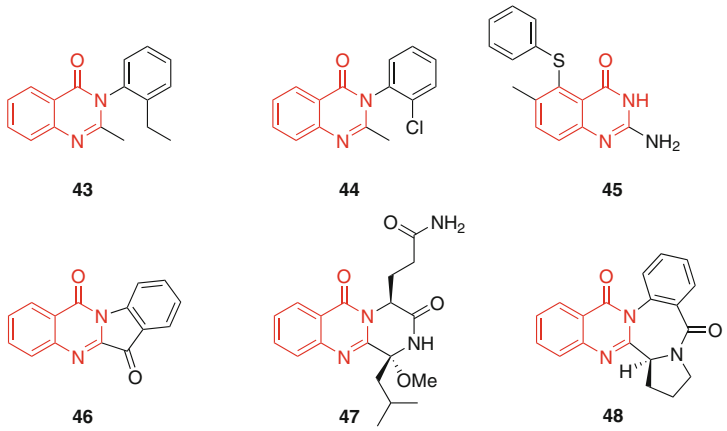


Fig. 26 Occurrence of the quinazolinone privileged scaffold (indicated in *red*) within the drugs Etaqualone (**43**, sedative), Mecloqualone (**44**, sedative), and Nolatrexed (**45**, antineoplastic) and the natural products Tryptanthrin (**46**, antiparasitic, found in various plants), Aurantiamide A (**47**, cytotoxic, isolated from *Penicillium aurantiogriseum*), and (−)-Circumdatin H (**48**, mitochondrial respiratory chain inhibitor, isolated from *Aspergillus ochraceus*)

A (CCK A), which is associated with gastrointestinal cancer (Evans et al. 1988). Combinatorial synthesis of a library of benzodiazepines by the Ellman group then led to the identification of a selective CCK A antagonist, Bz-423 (**42**, Bunin et al. 1994; Boitano et al. 2003).

It was then soon realized that many of such privileged structures appear across various drugs and natural products, often within completely different therapeutic areas. Various approaches such as NMR-based target binding analysis (Hajduk et al. 2000) and cheminformatics analysis of the CRC Dictionary of National Products (Koch et al. 2005) have led to a large list of privileged scaffolds, and it is likely that there are much more privileged structures out there that have not been identified yet. An extensive list of privileged structure prevalence was collected by Welsch and co-workers, of which a small part is shown in Fig. 26 to illustrate the concept (Welsch et al. 2010). Important to note is that the quinazolinone motive does not only have different targets but is also incorporated into natural products synthesized by completely different organisms. Of course, one could wonder why privileged structures have the ability to bind multiple targets and why nature makes frequent use of the same motives to synthesize natural products. This can be explained by the fact that, while there are a vast amount of proteins known, the types of structural features or folds that they incorporate are rather limited, resulting in similar binding sites even when the similarity of two proteins is not immediately obvious (Breinbauer et al. 2002). This similarity also implies that natural systems are likely to use similar strategies, e.g., for cellular signaling processes, defense against predators, and others (Welsch et al. 2010).

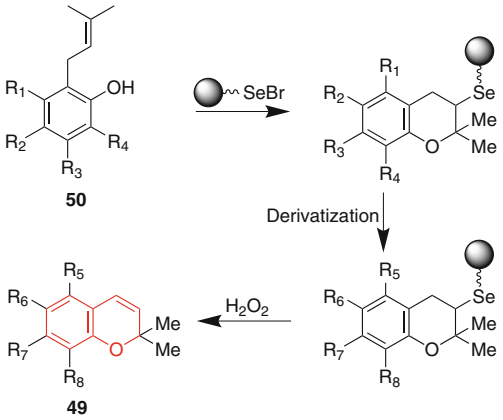


Fig. 27 Combinatorial approach to build a large library of natural-product-like compounds. Starting from butylenephenol **50**, the immobilized privileged scaffold are constructed on solid support and allow for the rapid generation of a large amount of variants containing the benzopyran moiety (indicated in *red*), which could be isolated by oxidative cleavage of the selenium-containing resin

By constructing privileged scaffolds on a solid support and then using combinatorial chemistry, various research groups have successfully built up libraries. Due to the large amount of research interest this topic has generated over the years, only a couple of examples will be discussed here.

A tremendous effort has been made by the Nicolaou group, who decided to build a library around the 2,2-dimethylbenzopyran (**49**) moiety. By attaching butylenephenol **50** to an oxidation-labile solid support, the privileged scaffold could be constructed, after which the residual groups were modified by exchanging functional groups or coupling additional fragments. This approach ultimately led to the construction of a library consisting of more than 10,000 different natural product-like compounds (Fig. 27) (Nicolaou et al. 2000).

In another example, privileged scaffolds were used by the Hergenrother group as starting point for a DOS approach, using so-called ring distortion reactions to generate new natural product-like scaffolds, in a concept they named "complexity to diversity" (CtB) approach (Huigens III et al. 2013). This idea is illustrated in Fig. 28. Starting from the natural steroid adrenosterone (**51**), five different scaffolds were synthesized in three to five stereoselective reactions in solution. These scaffolds were in turn further derivatized to yield a small library of structurally diverse and complex molecules. Using the same approach starting from the natural products gibberellic acid, quinine and abietic acid (Rafferty et al. 2014), a highly diverse and structurally complex library was attained.

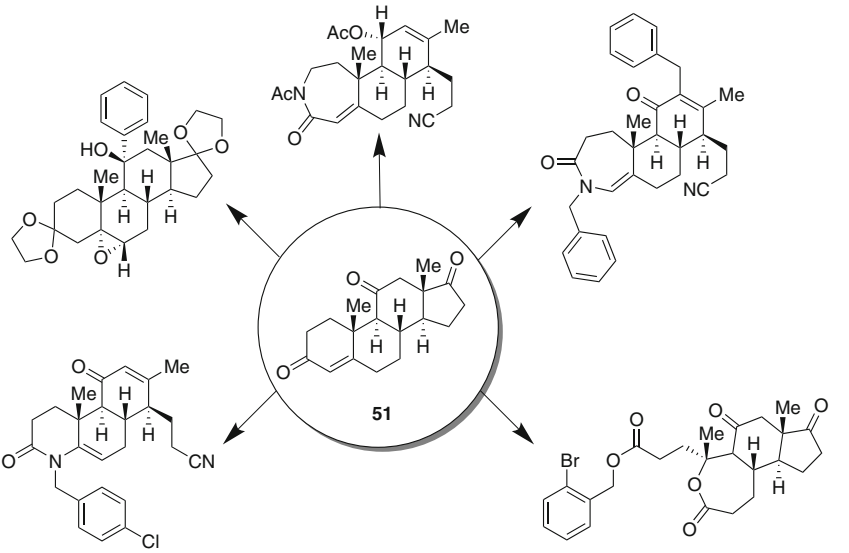


Fig. 28 Various scaffold variants to the natural steroid adrenosterone (**51**) synthesized through a ring distortion and DOS strategy in three to five steps as basis for a small library focused on diversity and complexity

4 Conclusion

Natural product research had its golden era basically from the early nineteenth century with the isolation of alkaloids and the exploration of mostly bacteria and fungi for new natural products in the mid-twentieth century. This still exerted a strong effect into our times of drug research. It is of course very clear that natural products have and still do contribute to men's health and life expectancy. They significantly contributed to the timely discovery of many cellular targets for drug development processes, and without these discoveries, progress in medicine would most probably still lag behind. There is also no doubt that there are still interesting and that important structures and bioactivities of new natural products can be found. They may even have groundbreaking properties to address new cellular targets and may provide cures we are currently unaware of. However, one has to draw a realistic picture on the developments in the sciences but also in the pharmaceutical industry. Many natural products or derivatives reaching the market in these days have been found decades ago and have been reconsidered for drug use because of their unique properties, which is evident with the antibiotics daptomycin, fidaxomicin, and ramoplanin. Fundamentally new natural products lead structures are scarce. Hence, the downscaling and termination of most natural product research groups within the pharmaceutical industry has its reasons. These comprise all the well-known arguments exchanged in a dispute between supporters and

opponents of natural product chemistry. A fact of our days is that the drug discovery aspect of natural product drugs has shifted to academic research groups and new, small companies that specifically cater to current unmet needs in the market, especially in the area of antibiotics. This seems to support the idea that the discovery aspect of drug development will be left into the hands of academics and small companies, while big pharmaceutical concerns will specialize into the developmental aspects as well as provide for the funds necessary for clinical investigations (Drews 2000).

An aspect supporting the above lines from an industrial perspective is that the number of principal biosynthetic pathways is limited. Fundamental principles, e.g., of polyketides synthases or nonribosomal peptide synthetases, have been discovered, and it seems unlikely that fundamentally new ones will be discovered. This means that the biochemistry is limited, and at some point, apart from the vast numbers of natural products which theoretically can be realized, their structural diversity has its limits. With the potential of the vast amount of bacterial, fungal, plant, and marine life species that have not yet been characterized and screened for bioactive compounds, it indeed remains to be seen whether with more genome data surprises will come up. However, a great perspective for future success will be to pursue engineering approaches in order to manipulate and engineer biosynthetic pathways.

On the other hand, chemical libraries have come a long way from large collections of random molecules with poor physiochemical properties and low hit rates to smaller libraries containing more structurally diverse- and complex compounds with more drug-like or natural product-like properties. While there is still an ongoing debate whether library construction from FBDD or DOS will yield more drug leads, both approaches have shown their merits as in the last decade we have seen a number of drug leads from FBDD and DOS libraries entering the market and clinical trials. Which approach to choose will be more likely dependent on what one hopes to achieve; FBDD is a target-oriented approach and requires a large amount of structural information about the target, which might not be suitable for exploring difficult to access targets where structural information is limited. On the other hand, DOS's high diversity could lead to some interesting surprises but could on the other hand also lead to huge libraries which are costly to maintain and might ultimately not render drug leads at all.

Either way, using privileged scaffolds from nature as starting points for FBDD or DOS library construction seems to be a promising way to go forward. As this field has generated a large amount of research interest over the past decade, drug leads originating from privileged structure libraries can be expected to start entering clinical research and the market over the course of this decade and the next. Furthermore, the ongoing efforts to identify new privileged structures in nature shows that natural product research still is relevant within the world of drug discovery.

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New Compound Classes: Protein–Protein Interactions

C. Ottmann

Contents

Abstract

"Protein–protein interactions (PPIs) are one of the most promising new targets in drug discovery. With estimates between 300,000 and 650,000 in human physiology, targeted modulation of PPIs would tremendously extend the "druggable" genome. In fact, in every disease a wealth of potentially addressable PPIs can be found making pharmacological intervention based on PPI modulators in principle a generally applicable technology. An impressing number of success stories in small-molecule PPI inhibition and natural-product PPI stabilization increasingly encourage academia and industry to invest in PPI modulation. In this chapter examples of both inhibition as well as stabilization of PPIs are reviewed including some of the technologies which has been used for their identification."

Keywords

Natural products • PPI inhibition • PPI stabilization • Small-molecules • X-ray crystallography

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Targeted modulation of protein–protein interactions (PPIs) with small, drug-like molecules is one of the most promising approaches in modern drug discovery (Arkin et al. 2014; Milroy et al. 2014). Not only the number of potentially addressable PPI targets with estimates between 130,000 and 650,000 (Venkatesan et al. 2008; Stumpf et al. 2008; Zhang et al. 2012) is significantly higher than those of single protein targets, but also a number of natural products and molecules from drug discovery initiatives illustrate that PPIs are addressable with small molecules. Both these facts mean that in principle every disease can be tackled by small-molecule PPI modulators, especially those that lack good, conventional targets like enzymes, GPCRs, ion channels, or nuclear receptors. In addition, for hard-to-target pathogenicities like Alzheimer's or metabolic diseases, PPI modulation may be a viable option definitely to be considered. However, despite these encouraging vantage points, identification and optimization of small-molecule modulators of PPIs still poses a formidable challenge.

1 Protein–Protein Interactions in Health and Disease

Direct physical interactions of proteins are essential to all processes in living organisms. One example is the reception and propagation of growth signals that can start with the binding of a proteinaceous signaling molecule like the epidermal growth factor to its cell-surface receptor. This ligand/receptor recognition event then triggers the assembly and activation of signaling complexes at the cytosolic site of the plasma membrane recruiting adapter proteins like Gbr2 and Sos as well as small G proteins like Ras. Ras then activates the Raf kinases, again mediated by direct physical interaction. In a subsequent phosphorylation cascade, Raf then stimulates other kinases (like MEK and ERK) that ultimately leads to gene activation via transcription factors (Wellbrock et al. 2004). As each of these steps necessitates direct binding of the proteins in this signal transduction chain, small molecules that bind to the involved interfaces of either one of these partners could disrupt this pro-proliferative signaling. Another principal possibility to attenuate such a pathway would be small-molecule stabilization of the binding of negative regulators to Raf (like RKIP, Zeng et al. 2008). The control of the spatial distribution of proteins is another important aspect of functional regulation performed by PPIs. For example, the pro-inflammatory transcription factor NFκB is prevented from nuclear import upon complexation with its negative regulator IκB (Shih et al. 2011). Stabilization of this protein complex could hence result in a therapeutic benefit in autoimmune diseases.

Direct regulation of biochemical activities of enzymes by PPIs is observed repeatedly. The phosphatase calcineurin, for example, is activated by binding to Ca^{2+}-activated calmodulin and repressed by cabin (*c*alcineurin-*b*inding prote*in*) or calcipressin (Liu 2009). Another important process involving PPIs is the functional constitution of transcriptional complexes. Although transcription factors of the Tcf/LEF family directly bind to DNA, transcription starts only when co-activators like β-catenin additionally interact with Tcf/LEF (Shitashige

et al. 2008). Many pathogens need host proteins as cofactors for their disease-causing activity. For example, exoenzyme S from *Pseudomonas aeruginosa*, an opportunistic bacterium that causes pneumonia, needs to interact with host 14-3-3 proteins to be able to transfer an ADP-ribose moiety from NAD^+ to small G proteins like Ras (Fu et al. 1993).

Many cellular functions like motility, e.g., in the context of angiogenesis, are related to functional changes in the cytoskeleton. The dynamic assembly and disassembly of actin filaments are based on the interaction of actin with itself and the binding to protein partners like cofilin and profilin (Bernstein and Bamburg 2010). Biological (surface) recognition, like in the immune system, is also mediated by PPIs as in the case of binding of LFA-1 (*l*ymphocyte *f*unction-associated *a*ntigen *1*) presented on the surface of immune cells to ICAM-1 (*i*ntracellular *a*dhesion *m*olecule *1*) found on the surface of endothelial cells (Lawson and Wolf 2009). This interaction enables immune cells to attach to the walls of blood vessels and to migrate into neighboring tissue to initiate inflammation.

2 Physiological Regulation of PPIs

Given the importance and number of PPIs in the living cell, it is no surprise that they have to be tightly regulated in a precise spatial and temporal manner. The occurrence and perseverance of PPIs are in general governed by the local concentration of the binding partners and the intrinsic binding energy of their interactions (Nooren and Thornton 2003). The first is regulated by (epi)genetic control and translational mechanisms, subcellular localization, proteolytic degradation, and temporary storage. The second is modulated by covalent modifications like phosphorylation, by changes in pH, ionic strength, and temperature. Furthermore, additional PPIs can influence binary interactions. These can lead to inhibition when, e.g., the interaction interface of one partner is masked by binding to the same interface or by simple sterical hindrance. PPIs can also be just induced by a further protein, for example, when the third interacting protein binds simultaneously to both protein partners. Such a "bridging" or "assembly platform" function has been described for the *A-k*inase *a*nchoring *p*roteins (AKAPs) (Wong, and Scott 2004) and the *k*inase *s*uppressor of *R*as (KSR) (Clapéron and Therrien 2007). It is clear that the local architecture of such signaling complexes is one of the keys to understand regulation and specificity of signaling events, and small-molecule intervention of these processes holds great promises for the development of novel therapeutic agents.

3 Small-Molecule Modulation of PPIs: Inhibition

An early example of an FDA-approved (1999) PPI inhibitor is **tirofiban** (trade name Aggrastat®, Merck & Co.) (Hartman et al. 1992; Springer et al. 2008). Tirofiban was designed as an RGD tripeptide mimic that binds to the integrin

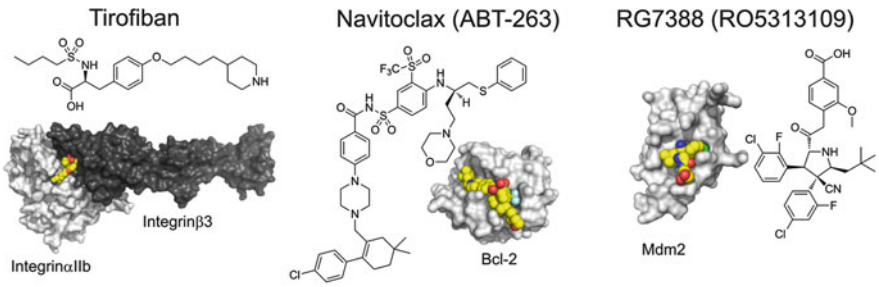

Fig. 1 Small-molecule inhibitors of extracellular integrin's binding to fibrinogen, anti-apoptotic Bcl-2's (B-cell lymphoma 2) binding to pro-apoptotic Bik (Bcl-2 interacting killer), and Mdm2's (mouse double minute 2) binding to p53. The PDB codes of the crystal structures are 2VDM, 4LVT, and 4JRG, respectively

IIa/IIIb and prevents interaction with the RGD motif of fibrinogen, thus displaying antithrombotic activity (Hartman et al. 1992). There are a number of PPI inhibitors that are quite advanced in clinical development. For example, the Bcl2/BclXL inhibitor **navitoclax** (ABT-263) developed by Abbott Laboratories (Tse et al. 2008) is right now undergoing several clinical phase I/II trials for the treatment of diverse cancers (Cleary et al. 2014; Rudin et al. 2012; Roberts et al. 2012). **RG7388**, developed by Roche, is an inhibitor of the interaction of the tumor suppressor p53 with its negative regulator HDM2 and is currently undergoing clinical trials (Fig. 1) (Ding et al. 2013).

Likewise, several inhibitors of IAP (inhibitor of apoptosis) proteins are currently tested in phase I/II clinical trials (Fulda 2015). SMAC (second mitochondrial activator of caspase) is an endogenous negative regulator of IAPs that liberate caspases from inhibition by IAPs, thus triggering apoptosis (Fulda and Vucic 2012). Small-molecule IAP inhibitors mimic the tetrapeptide Ala-Val-Pro-Ile (AVPI) of Smac that mediates the binding to IAP (Liu et al. 2000). Since SMACs have been reported to bind as homodimers to the IAP interaction domains BIR2 and BIR3 (Huang et al. 2003), both monovalent and bivalent SMAC mimetics have been developed. Examples for monovalent IAP inhibitors are **GDC-0152** from Genentech (Erickson et al. 2013) and **LCL-161** from Novartis (Dubrez et al. 2013). A bivalent inhibitor is, for example, **birinapant**, developed by TetraLogic (Benetatos et al. 2014). The bivalent antagonist shows better cellular potency but has to be administered intravenously, in contrast to the orally available monovalent IAP inhibitors.

Another group of clinical candidate molecules that attracted immense attention in recent years are inhibitors of bromodomains that bind to recognition motifs that contain an acetylated lysine and that are developed as novel agents for the therapy of cancer, inflammation, diabetes, and viral infection (Filippakopoulos and Knapp 2014). For example, **I-BET726**, developed by GSK, binds with high affinity (4 nM)

to the BRD4 bromodomain and shows potent activity in murine models of neuroblastoma (Gosmini et al. 2014). **CPI-0610** from Constellation Pharmaceuticals inhibits bromodomain 1 (BRD1) from BRD4 with an IC50 of 29 nM (Gehling et al. 2013) and is currently in a clinical trial as treatment of progressing lymphoma (Filippakopoulos and Knapp 2014). **RVX-208** from Resverlogix is a bromodomain inhibitor that shows selectivity for binding to the second bromodomain (BRD2) (Picaud et al. 2013) and that causes a reduction of atherosclerosis in hyperlipidemic ApoE-deficient mice (Jahagirdar et al. 2014).

In total, there are more than 50 PPIs that have successfully been targeted with small molecules, as described recently in an update of the TIMBAL database (Higueruelo et al. 2013). Most of these are inhibitors of PPIs, disrupting binary protein complexes like CD80/CD28 (Uvebrant et al. 2007), XIAP/caspase9 (Sun et al. 2010), and HIF-1α/p300 (Kwon et al. 2012), but also the opposite strategy – to stabilize a given PPI – is represented in this database, e.g., stabilizers of the 14-3-3/C-Raf (Ottmann et al. 2009; Molzan et al. 2013). Although the latter approach is still underrepresented in drug discovery and chemical biology, a growing number of examples illustrate its feasibility (for review, see Milroy et al. 2014; Zinzalla and Thurston 2009; Thiel et al. 2012; Jin et al. 2014; Giordanetto et al. 2014).

As might be guessed and already discussed in the examples above, especially pharmaceutical companies identified PPI modulators. One of the most popular PPI targets to be inhibited is without doubt the p53/HDM2 complex. In addition to the already mentioned discovery of the nutlins by Roche (Vassilev et al. 2004), at Amgen an internal library of 1,400,000 compounds was screened via HTRF (Allen et al. 2009), and at Johnson & Johnson a ThermoFluor assay was employed to identify such inhibitors from a 338,000-compound library (Koblish et al. 2006). However, also academic groups contributed molecules addressing this target, for example, the groups of Wang (Shangary et al. 2008) and Doemling (Czarna et al. 2010), who used in silico methods to discover p53/HDM2 inhibitors (Fig. 2).

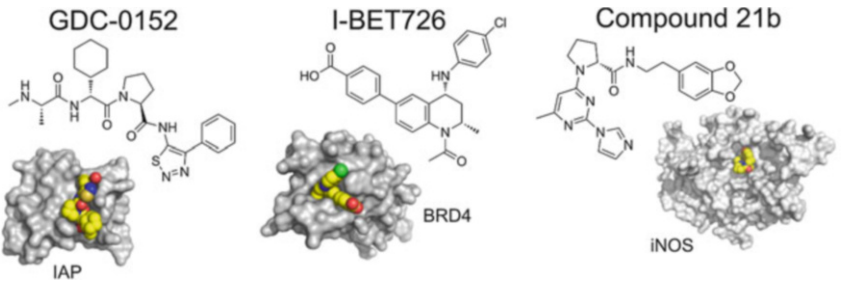

Fig. 2 Small-molecule inhibitors of IAP (inhibitor of apoptosis) binding to pro-apoptotic caspases, BRD4 (bromodomain-containing protein 4) to, for example, histones, and homodimerization of iNOS (inducible nitric oxide synthase). The PDB codes of the crystal structures are 3UW5, 4UYF, and 2ORO, respectively

Another target that raised a lot of attention is homodimerization of iNOS. Here, companies like Berlex Biosciences (Davey et al. 2007) and Adolor Corporation (Chu et al. 2009) reported discovery and optimization of disruptors of the iNOS/iNOS complex. **Compound 21b** from Berlex significantly ameliorated adjuvant-induced arthritis in a rat model and could be crystallized in complex with iNOS. **Compound 6** developed by Adolor is a potent inhibitor of iNOS dimerization in a cell-based iNOS assay (IC50 = 12 nM).

Human papillomavirus (HPV) is implicated in causing cervical cancer, the second most prevalent cancer in women worldwide. Here, the interaction of the viral proteins E1 and E2 as well as E6 with the host cell ubiquitin ligase E6AP has been targeted with small-molecule PPI inhibitors (D'Abramo and Archambault 2011). Researchers at Boehringer Ingelheim identified inhibitors of the herpes virus E1/E2 protein complex from a library of 140,000 compounds (Yoakim et al. 2003). Inhibitor 1 (**BILH434**) derived from this study displayed an IC50 of 0.18 μM and was crystallized in complex with E2 (Wang et al. 2004). Recently, a study was published identifying compounds from a library screen against the E6/E6AP interaction (Malecka et al. 2014). From the 30 validated hits (library size 88,000), seven inhibited p53 degradation in cell lines with HPV-integrated genomes, and two of these blocked p53 degradation and inhibited cell proliferation in cells specifically and stably transfected with E6. Progression through the cell cycle is among others regulated by the cyclin-dependent protein kinases (Cdks) with overactivation of these kinases being associated with the development of cancer (Murray 2004). Hence, inhibition of the stimulatory Cdk/cyclin interaction could be one strategy for anticancer therapy. At Merck Research Laboratories, a 5,000,000-compound library was screened to identify inhibitors of the Cdk2/cyclin A interaction (Deng et al. 2014). Quinoline-based **compound 2** bound to Cdk2 with a Kd of 0.3 μM and was crystallized in complex with Cdk2. The molecule binding to the kinase domain induced a conformational change mainly involving helix C that is incompatible with binding of cyclin A. At Wyeth Research, inhibitors of the ZipA/FtsZ complex were found by screening 250,000 compounds using a fluorescence polarization (FP) assay (Kenny et al. 2003). **Pyridopyrimidine 1 inhibitor** from this screen showed a K_I of 12 μM and could be co-crystallized with ZipA. This structure was used in a subsequent study for a scaffold hopping approach which produces new leads for further development (Rush et al. 2005). In a third, complementary approach at Wyeth, NMR fragment screening was employed to find novel inhibitors of the ZipA/FtsZ complex (Fig. 3) (Tsao et al. 2006).

Also, more and more academic groups involve themselves in the identification and development of PPI modulators as compound libraries and screening facilities become more available for noncommercial entities, too. For example, researchers at the FMP in Berlin, Germany, identified inhibitors of the clathrin terminal domain (CTD)/amphiphysin interaction named **pitstops 1** and **2** using an ELISA-based assay (von Kleist et al. 2011). The group of Wagner found inhibitors of the translation initiation factor eIF4E/eIF4G complex in a 16,000-compound library screened employing FP (Moerke et al. 2007). In addition to the use of disruptors of this complex as tool compounds to study processes such as cell growth, embryonic

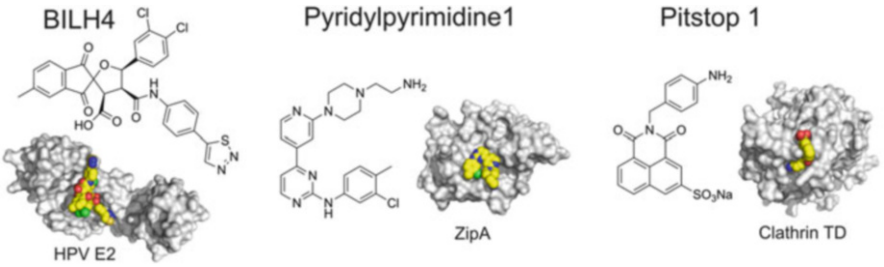

Fig. 3 Small-molecule inhibitors of HPV E2 (human papillomavirus E2 protein) binding to HPV E1, interaction of bacterial ZipA (Z interacting protein A) with FtsZ (filamenting temperature-sensitive mutant Z), and CTD's (clathrin terminal domain) binding to amphiphysin. The PDB codes of the crystal structures are 1R6N, 1Y2F, and 2XZG, respectively

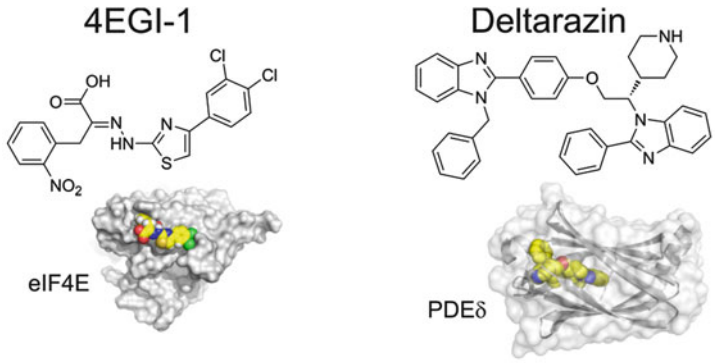

Fig. 4 Small-molecule inhibitors of eIF4E's (eukaryotic translation initiation factor 4E) binding to eIF4G and of PDEδ's (phosphodiesterase delta subunit) interaction with KRAS (Kirsten rat sarcoma). The PDB codes of the crystal structures are 4TQC and 4JVF, respectively

development, apoptosis, synaptic plasticity, and axon guidance, the authors suggest that they could also represent a new strategy for small-molecule cancer therapy. Their most active compound – **4EGI-1** – was just recently crystallized in complex with eIF4E revealing an allosteric mechanism of PPI inhibition (Papadopoulos et al. 2014). The group of Wilding found inhibitors of the androgen receptor (AR)/JunD interaction by screening a 27,000-compound library with a luciferase complementation assay (Mehraein-Ghomi et al. 2014). Inhibiting the AR/JunD complex is expected to reduce production of carcinogenic reactive oxygen species (ROS) in prostate epithelial cells. Their best hit – **GWARJD10** – significantly reduced androgen-induced transcriptional activity. The group of Waldmann reported the identification and medicinal chemistry optimization of inhibitors of the PDEδ/KRAS interaction by screening a compound library with an AlphaScreen assay (Fig. 4) (Zimmermann et al. 2013).

Starting from an already promising benzamidine hit compound that bound to PDEδ with a Kd of 217 nM (isothermal titration calorimetry) and guided by the co-crystal structure that revealed two molecules binding to the hydrophobic tunnel in PDEδ, an optimized compound (**deltarasin**) was developed that bound PDEδ within the cell with a Kd of 41 nM. Deltarasin showed suppression of proliferation in KRAS-dependent pancreatic ductal adenocarcinoma cell models and thus provides a new opportunity to suppress oncogenic Ras signaling and tumor growth.

The group of Gestwicki recently reported the identification of inhibitors of Hsp70 employing an ATPase assay on a 55,400-compound library (Miyata et al. 2010). Interestingly, Gestwicki and co-workers also identified an activator of ATPase activity in *E. coli* Hsp70 that could be converted into an inhibitor of the Hsp70/Hsp40 interaction (Wisén et al. 2010). The first screen identified the compound **115-7c** that showed a higher affinity to the Hsp70/Hsp40 complex than to Hsp70 alone, implying that 115-7c might bind to a composite site in the complex. Guided by docking experiments, a more bulky derivative of 115-7c was synthesized (**116-9e**) that was found to inhibit the Hsp70/Hsp40 interaction. This example nicely illustrates that in principle a molecule that binds to an interface pocket of a protein complex and might stabilize the interaction can be converted into an inhibitor by introducing sufficient potential for steric clashes. It remains an interesting possibility that this principle in turn might be applied to create a PPI stabilizer from known interface inhibitors.

4 Small-Molecule Stabilizers of PPIs

Quite recently, also a number of stabilizers, sometimes called inducers of PPIs, have been described. Among them, **bioymifi** leads to specific aggregation of the extracellular domain of the death receptor DR5 (Wang et al. 2013), and **chlorazol violet N** has been reported to be a partial agonist of the OX40/OX40L interaction (Song et al. 2014). In an ELISA-based assay, we have previously identified the molecules **epibestatin** and **pyrrolidone 1** as stabilizers of the 14-3-3/PMA complex from a 37,000-compound library (Rose et al. 2010). At Roche, researchers used HTRF to screen for inhibitors of the MDMX/p53 interaction and actually identified a molecule (**RO2443**) that stabilizes the MDMX homodimer (Graves et al. 2012). Since the RO2443-stabilized HDMX/HDMX interface includes the binding site for the p53 interaction, this molecule is an effective functional inhibitor of the negative regulation of p53 by HDMX. Four more PPI-stabilizing small molecules have also been identified in screens for inhibitors and in fact act as stabilizers of inactive, oligomeric states of their target protein (Fig. 5).

Trifluoperazine (TPA) induces and stabilizes a pentameric state of the cancer-related protein S100A4 (Malashkevich et al. 2010); **BMS-883559** stabilizes the inactive hexamer of the nucleoprotein InfNP from the influenza virus (Gerritz et al. 2011); **dexrazoxane** (ICRF-187) stabilizes the topoisomerase II homodimer,

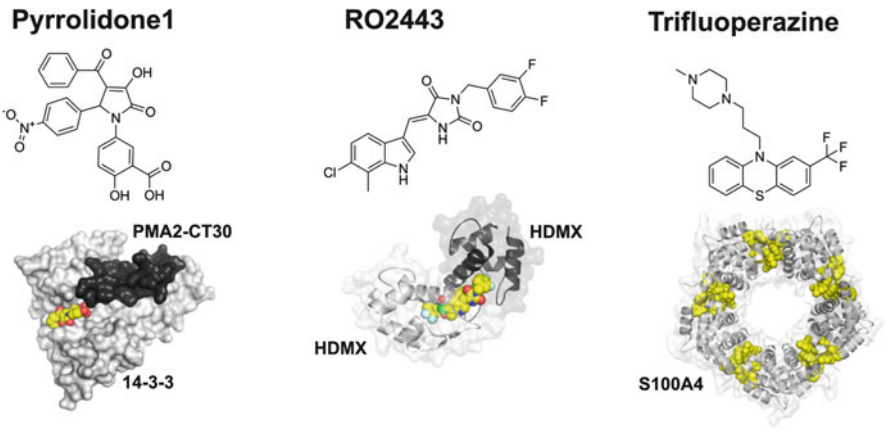

Fig. 5 Small-molecule stabilizers of 14-3-3's interaction with PMA2-CT30 (plasma membrane ATPase 2-C-terminus 30), HDMX's (human double minute X) homodimerization, and S100A4 oligomer. The PDB codes of the crystal structures are 3M51, 3U15, and 3K0O, respectively

thus inhibiting its function (Classen et al. 2003); and **tafamidis** works as a stabilizer of the tetrameric form of transthyretin (Bulawa et al. 2012). Another heterodimeric complex-stabilizing compound is the surprisingly simple molecule **NS309** that enhances the affinity of calmodulin (CaM) to the respective binding domain (CaMBD) of the SK potassium channel (Fig. 6) (Zhang et al. 2013).

5 Conclusions and Outlook

In this chapter, a number of success examples of inhibition as well as stabilization of PPIs illustrate the feasibility of this approach in drug development. With an estimated number between 130,000 and 650,000 PPIs in the human body, in principle it is plausible to identify a "druggable" PPI for every disease or pathological condition. Since nature itself regulates protein function very often by controlling interactions among proteins, the strategy to modulate PPIs with small molecules is a promising concept to complement more classical approaches of pharmacological intervention. As reviewed here, inhibition of PPIs is by far the prevalent strategy, clearly favored by the beautiful successes of molecules like tirofiban, navitoclax, nutlins, or bromodomain inhibitors. However, PPI-stabilizing compounds display a number of generally advantageous features that make them ideal complements to active site inhibitors and disruptors of PPIs. One important characteristic is their uncompetitive nature. As far as we know, PPI stabilizers don't have to compete neither with a small molecule nor another biomacromolecule (protein, complex carbohydrate, DNA, or RNA) for their binding pocket. Especially in the immensely crowded intracellular environment, this is helpful for achieving a physiologically significant activity on their targets. Related to this, a

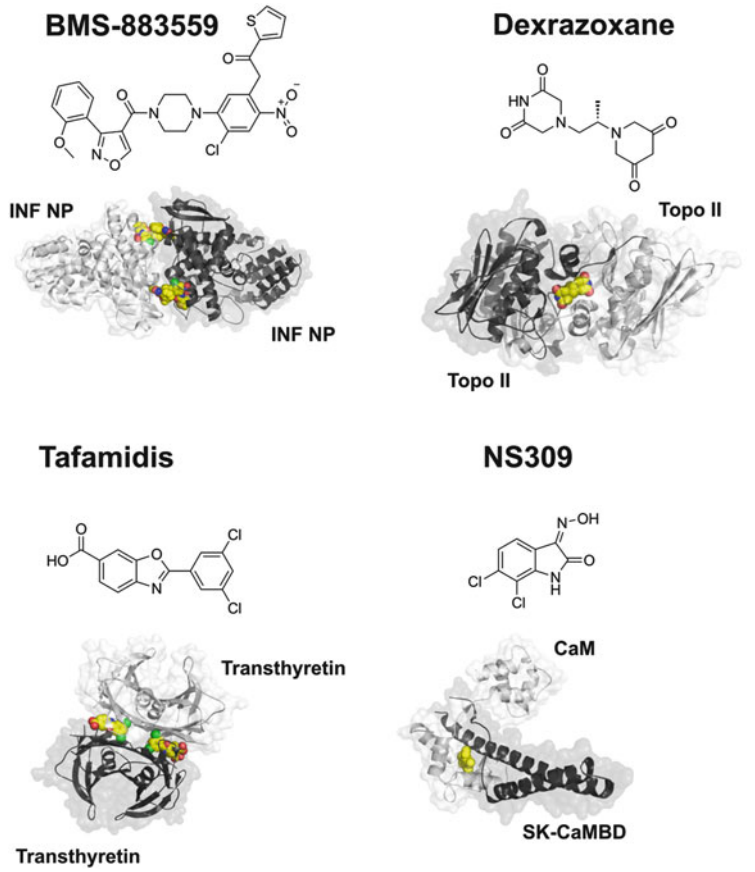

Fig. 6 Small-molecule stabilizers of the INF NP (influenza nucleoprotein) homodimer, topo II (topoisomerase II) homodimer, transthyretin homotetramer, and the complex of SK-CaMBD (small-conductance K channel calmodulin-binding domain) and CaM (calmodulin). The PDB codes of the crystal structures are 4DYB, 1QZR, 3TCT, and 4J9Z, respectively

second advantage is of thermodynamic nature. In general, it can be expected that the amount of binding energy that has to be added by a small-molecule binding event to an already existing and biologically relevant protein complex is much less than the energy that is needed to disrupt a given protein–protein or protein–ligand interaction. This in turn would allow for a weaker overall affinity of the stabilizing ligand in comparison to an inhibitor of an active site or a PPI interface.

In the last 5–10 years, our knowledge on how to target PPIs with small molecules has dramatically increased, thus holding great promises for the clinical application of this kind of compounds.

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Using Cheminformatics in Drug Discovery

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Abstract

This chapter illustrates how cheminformatics can be applied to designing novel compounds that are active at the primary target and have good predicted ADMET properties. Examples of various cheminformatics techniques are illustrated in the process of designing inhibitors that inhibit both cyclooxygenase isoforms but are more potent toward COX-2. The first step in the process is to create a knowledge database of cyclooxygenase inhibitors in the public domain. This data was analyzed to find activity cliffs – small structural changes that result in drastic changes in potency. Additional cyclooxygenase potency and selectivity trends were obtained using matched molecular pair analysis. QSAR models were then developed to predict cyclooxygenase potency and selectivity. Next, computational algorithms were used to generate novel scaffolds starting from known cyclooxygenase inhibitors. Nine virtual libraries containing 240 compounds each were constructed. Predictions from the cyclooxygenase QSAR models were used to eliminate molecules with undesirable potency or selectivity. Additionally, the compounds were screened in silico for undesirable ADMET properties, e.g., low solubility, permeability, metabolic stability, or high toxicity, using a liability scoring system known as ADMET Risk™. Eight synthetic candidates were identified from this process after incorporating knowledge gained from activity cliff analysis. Four of the compounds were synthesized and tested to measure their COX-1 and COX-2 IC_{50} values as well as several ADME properties. The best compound, SLP0020, had a COX-1 IC_{50} of 770 nM and COX-2 IC_{50} of 130 nM.

Keywords

Activity cliffs • ADMET • Combinatorial design • COX-1 • COX-2 • Cyclooxygenase • Drug design • Matched molecular pairs • QSAR • QSPR • Scaffold hopping

1 Introduction

Traditionally, the term "drug discovery" has brought to mind robots conducting automated assay screens on thousands of molecules. There are faster and cheaper alternatives, however, particularly when a substantial amount of information about the target of interest already exists. This chapter focuses on how to discover drug leads in silico by judicious application of cheminformatics. The ultimate goal is to identify novel compounds that are potent at the primary target and have good absorption, distribution, metabolism, excretion, and toxicity (ADMET) properties.

We will use examples from one of our in-house proof-of-concept discovery projects, where the objective was to design inhibitors that discriminate between the two isoforms of cyclooxygenase, COX-1 and COX-2 (Penning et al. 1997; Maxwell and Webb 2005). Starting from a knowledge database of IC_{50} values for about 550 compounds that was compiled from the public literature, we built predictive

quantitative structure activity relationship (QSAR) models and applied them to combinatorial libraries built around scaffolds that had not been thoroughly explored.

Candidates from those virtual libraries were screened in silico for activity, selectivity, and ADMET liabilities. A few of the most attractive candidates were synthesized and tested in relevant biological assays, and their ADMET properties were measured; all were active and ADMET properties were in reasonable agreement with prediction.

2 Proof-of-Concept Target Selection

Cyclooxygenase is involved in the production of prostaglandins, which mediate a wide range of biological responses, including inflammation, constriction and dilation of vascular smooth muscle, aggregation of platelets, and regulation of gastric acid secretion. All COX isoforms convert arachidonic acid into prostaglandin H_2 (PGH_2) which can then be converted to other prostaglandins such as PGD_2, PGE_2, PGI_2, (prostacyclin), and TXA_2 (thromboxane) by other enzymes. The COX-1 isoform is constitutively expressed in many tissues and is the predominant form in the gastric mucosa and kidneys. Inhibition of COX-1 reduces levels of PGE_2 and PGI_2 which can contribute to gastric ulceration. COX-2 is an inducible isoform of cyclooxygenase that is elevated during inflammation.

When COX-2 was discovered in the 1990s, pharmaceutical companies raced to develop COX-2 inhibitors that were selective over COX-1 in order to alleviate pain without the risk of gastric problems associated with COX-1 inhibition. The compounds shown in Fig. 1 are COX-2-selective inhibitors that were approved in the late 1990s and early 2000s.

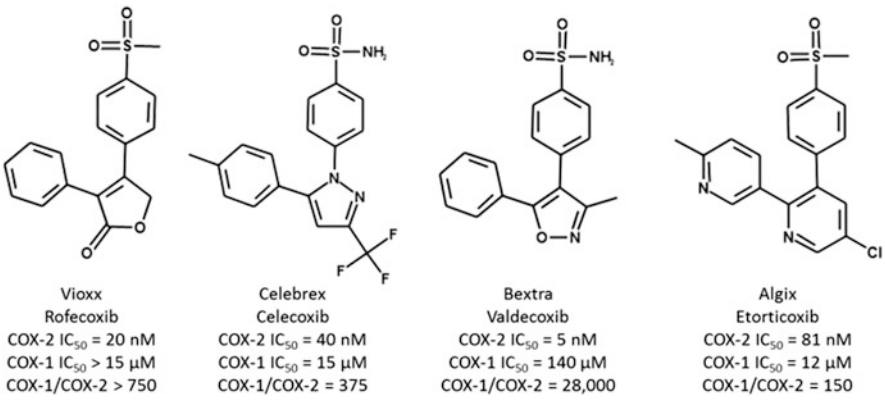


Fig. 1 Literature COX-1 and COX-2 IC_{50} values and selectivities for COX-2-selective inhibitors: Vioxx (Prasit et al. 1999), Celebrex (Penning et al. 1997), Bextra (Talley et al. 2000), and Algix (Friesen et al. 1998). Vioxx and Bextra were removed from the US market. Algix is approved in Europe but not in the USA

In 2001, Merck began a 3-year clinical trial called "APPROVe" (Adenomatous Polyp PRevention On Vioxx) to evaluate the efficacy of rofecoxib (the active ingredient in Vioxx) for preventing colorectal cancer. The study was terminated after 18 months because Vioxx increased adverse cardiovascular events compared to placebo. Merck then voluntarily withdrew rofecoxib products from the market.

This result has subsequently been interpreted as indicating that highly selective COX-2 inhibitors upset the balance of prostaglandins that is needed for vascular health (Maxwell and Webb 2005). Indeed, after withdrawing Vioxx from the market, Merck registered Algix in Europe. Its active ingredient – etoricoxib – has a better selectivity profile and the same cardiovascular risk as the nonselective diclofenac (Conaghan 2012; Cannon et al. 2006). We set out to identify other molecules that were selective for COX-2 but still inhibited COX-1 enough to provide an optimal prostaglandin balance.

3 Design Strategy

Figure 2 shows the overall strategy we used to design cyclooxygenase inhibitors. The first step in the process was to collect structures and inhibition data from the public domain. This data was surveyed manually to generate hypotheses about the structural requirements for cyclooxygenase inhibition.

It is also important to understand the structural changes that convert an *active* molecule into an *inactive* one or vice versa. One way to do this is by identifying "activity cliffs," i.e., small structural changes that result in a drastic change in potency (Maggiora 2006). We also used the related technique of matched molecular pair analysis (MMPA) to explore the effects of a given structural change on

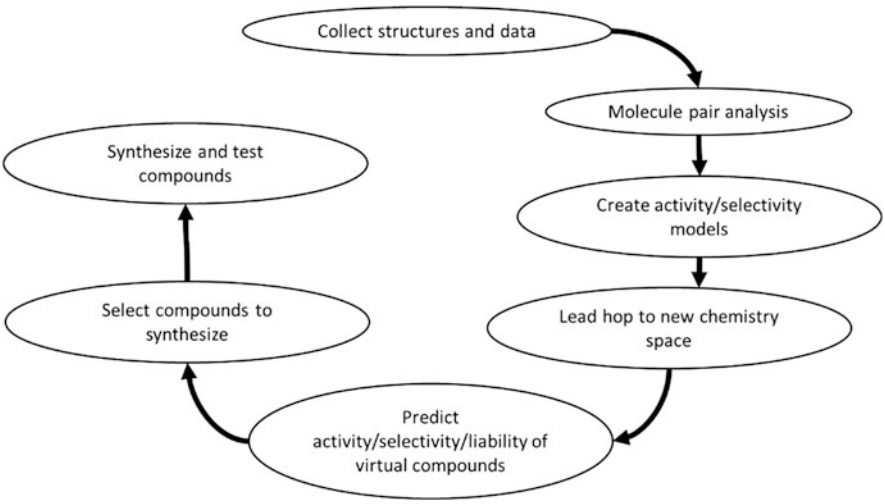


Fig. 2 Strategy for design of cyclooxygenase inhibitors

inhibitory activity as well as *selectivity* with respect to the two cyclooxygenase isoforms.

Matched molecular pair analysis relates structural change to activity change on a qualitative basis. Three QSAR models were also generated for the COX isoforms. The first was a classification model for predicting which compounds would exhibit COX-2 IC_{50} below 10 μM. The second and third were regression models for predicting the actual inhibitory potency against COX-1 and COX-2.

These steps were followed by scaffold hopping into novel chemistry space and subsequent elaboration into virtual libraries for which a range of properties were predicted. Here, a battery of ADMET models from ADMET Predictor™[1] were used to predict physicochemical (solubility, logP, pK_a, etc.), pharmacokinetic (plasma protein binding, blood/plasma concentration ratio, fraction unbound in plasma, and volume of distribution), metabolic (high intrinsic clearance and CYP 3A4 inhibition), and toxicological (hERG inhibition, carcinogenicity, liver injury, etc.) properties for the compounds that were predicted to be sufficiently active. Compounds that violated ADMET property thresholds determined from a reference subset of the World Drug Index (WDI) were penalized. A list of synthesis candidates was then constructed by manual inspection of the 240 that remained in the library. Those that were successfully synthesized were then submitted for biological testing and limited ADMET property determination.

4 Computational Methods

4.1 Data Set

While much literature on cyclooxygenase inhibitors has appeared since 1990, we focused on the data reported in articles published by either Searle (which was eventually acquired by Pfizer) or Merck to provide a starting point for ligand-based design using a few hundred compounds. The actual molecular structures together with COX-1 and COX-2 IC_{50} values were extracted from several sources including the ChEMBL database (Gaulton et al. 2012) and a published compilation (Chavatte et al. 2001) as well as from the primary literature.

4.2 Extracting Data from ChEMBL

The ChEMBL database can be extremely helpful for creating knowledge databases because it makes structure, biological data, and journal references for a given target accessible from a single source. The steps used to extract our COX-2 data from

[1] ADMET Predictor™ is distributed by Simulations Plus, Inc., Lancaster CA, http://www.simulations-plus.com.

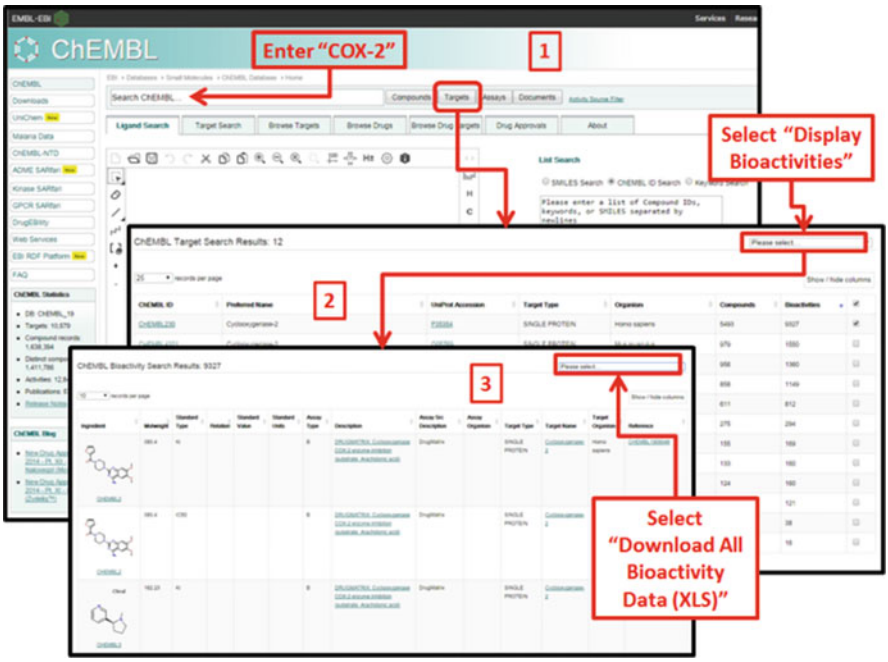


Fig. 3 Illustration of COX-2 structures and data downloaded from ChEMBL after entering "COX-2" as the query, displaying the bioactives, and then downloading the data as an XLS file. All data from the cyclooxygenase knowledge database was incorporated into a MedChem Studio "CTK" file

ChEMBL – outlined below and illustrated in Fig. 3 – provide an instructive example of the general procedure. We performed the following steps:

1. Opened an Internet browser and navigated to[2] https://www.ebi.ac.uk/chembl.
2. Entered "COX-2" for the "Search ChEMBL. . ." query.
3. Clicked on the "Targets" button (in our case, this resulted in 12 "hits").
4. Selected the record containing results for "Homo sapiens" and unchecked all other records.
5. Selected "Display Bioactivities" from the pull-down menu. This loaded a new Web page containing structures and data.
6. Selected "Download All Bioactivity Data (XLS)" on the next screen.
7. Specified a file name and location for the output Excel spreadsheet.

The Excel spreadsheet generated by the ChEMBL website contains several helpful fields. The "COMPOUND_KEY" is the identifier used in the journal article allowing one to quickly find the structure or data within the article.

[2] This section refers to the site as it existed in December of 2014.

The structure of a compound is provided as a SMILES (Weininger 1988; Weininger et al. 1989) string in the CANONICAL_SMILES column. "STANDARD_TYPE" indicates the data type, e.g., IC_{50}, Inhibition, or EC_{50}. "DESCRIPTION" gives a brief explanation of the assay. For COX-2 assays, this field indicates whether the assay was performed in whole blood or not, which provides critical context for the associated IC_{50} value. The journal reference spans five fields (JOURNAL, YEAR, VOLUME, ISSUE, and FIRST_PAGE) that can be combined into a unified "Reference" field. The Excel spreadsheet can then be manipulated and edited to create a customized structure file for import into software programs like MedChem Studio™.[3]

Including the compound identifier from the literature source as part of the name in the knowledge database and numbering references sequentially for inclusion in the compound name is a useful bookkeeping technique. For example, compound **3–100** refers to compound **100** from data set 3. Including IC_{50} values and their "modifiers" (=, >, or <), the journal reference, pharmaceutical company name, and a hyperlink to a PDF of the journal article is also helpful. Links to PDF files are useful when curating data because they facilitate rapid access to the relevant journal article to check the structure and property data. In some cases, critical contextual details are provided for interpreting the experimental data.

Cyclooxygenase pIC_{50} (negative log IC_{50} [M]) and selectivity (COX-1 IC_{50}/COX-2 IC_{50}) values were then calculated for each compound using MedChem Studio.

5 Activity Cliff Detection and Matched Molecular Pair Analysis

Gerald Maggiora helped introduce the concept of "activity cliffs" (Maggiora 2006), which are essentially small structural changes in molecules that result in drastically disproportionate changes in some biological activity or physicochemical property. Each pair of molecules in the data set is analyzed, and their structural difference is compared with the difference in the desired property. The pairs are then filtered to remove cases where the structural difference is larger than a specified similarity threshold or the difference in activity is smaller than a second threshold.

MedChem Studio was used to perform this analysis in order to find COX-2 activity cliffs in our data set. Figure 4 illustrates the general considerations involved in an activity cliff analysis. Thresholds for structural (dis)similarity are specified in terms of the minimum Tanimoto coefficient using substructural fingerprints, the maximum number of bond/atom differences (editing distance), and the maximum allowed number of sites where the structures may differ.

Clicking the "OK" button on the molecular pair generation dialog box opens the "Molecular Pairs" spreadsheet. An example is shown in Fig. 5. The structures shown

[3] MedChem Studio™ is distributed by Simulations Plus, Inc., Lancaster CA, http://www.simulations-plus.com.

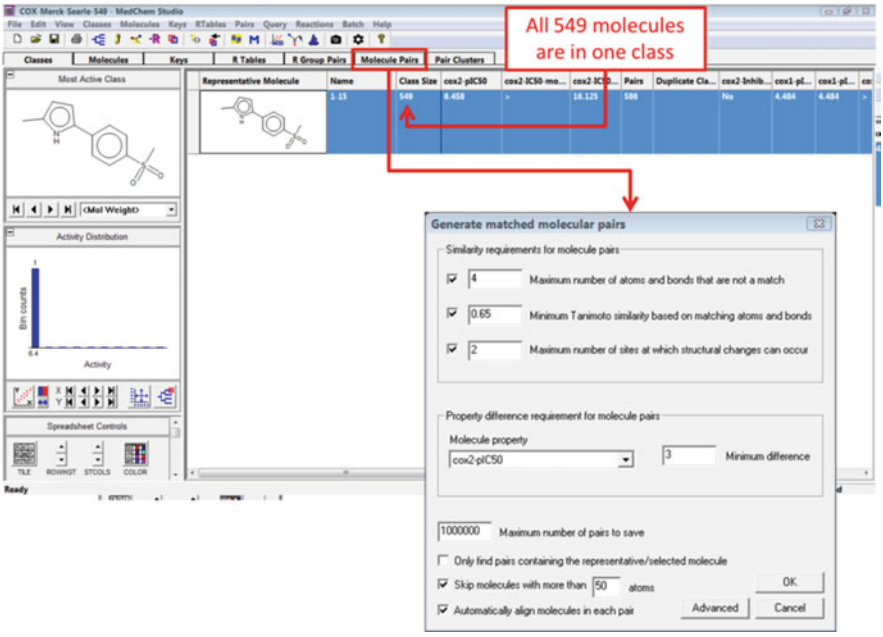


Fig. 4 Identifying activity cliffs in MedChem Studio for a particular structural class. Similarity between the pairs of molecules is indicated at the top of the dialog box, and the selected property to analyze is in the middle of the dialog box

for the molecules in each pair are aligned by their common scaffold with the differences between them highlighted in red in the molecule on the left and in blue in the molecule on the right. Aligning the scaffolds makes it easier to see the difference between them, as does highlighting the atoms and bonds that distinguish the two.

Each molecular pair represents a virtual reaction in which one or a few substituents on a shared scaffold are replaced with another set of substituents. In matched molecular pair analysis (MMPA), molecular pairs are grouped on the basis of that virtual reaction i.e., by grouping the *differences* between substituents within the pairs. For example, consider 20 pairs where the molecules in each pair differ *only* by replacement of an aromatic hydrogen in one molecule with a hydroxyl group in the other. This allows the average change in properties (solubility, logP, etc.) associated with a specific chemical transformation across the pairs to be computed as well as the distribution of differences around that average. This analysis thus offers insight into the degree of change in the various properties to be expected when this transformation is applied to a new scaffold.

MMPA generally starts the same way as generating activity cliffs. The difference is that pairs that have the same transformation are grouped together without filtering out those cases where the change in some specific property is small. The next step in the process is to analyze the property changes associated with a specific transformation. As an example, the specific steps used to assess the general effect of replacing a methyl sulfone group with a primary sulfonamide in MedChem Studio

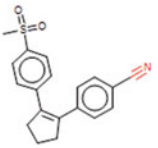
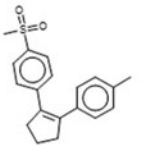
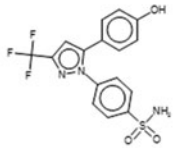
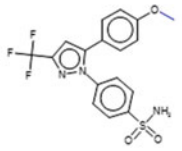
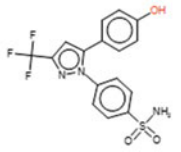
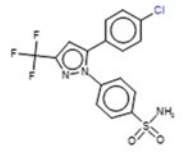
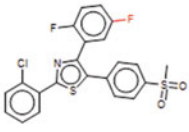
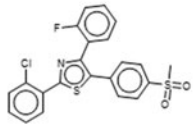
Index	Molecule 1	Molecule 2	cox2-pIC50 1	cox2-pIC50 2	Change(cox2-pIC50)
0			4.108	8.523	4.414
1			4.000	8.097	4.097
2			4.000	8.000	4.000
3			4.000	8.000	4.000

Fig. 5 Molecular pair spreadsheet showing molecular pairs along with the COX-2 pIC50 values of each compound and the difference (change) in activity between the two molecules

are outlined in the workflow below (refer to the dialog box shown in Fig. 4). A "class" in MedChem Studio is allowed to contain any number of molecules, including only one. They can be created in many ways, e.g., user definitions or clustering algorithms within the program.

1. Create a single class containing all molecules of interest.
2. Click the "Molecule Pairs" tab and set values for (or use default values):
   a. The maximum number of atoms and bonds that do not match within a pair.
   b. Minimum Tanimoto similarity based on matching atoms and bonds.
   c. The maximum number of sites at which structural changes can occur.
   d. A primary molecular property of interest (e.g., COX2 pIC50).
3. Select "Pairs → Reaction Query." This brings up a dialog box.
4. Enter the SMIRKS string as the "Transformation Reaction," e.g.,

$$[S:1](=[O:2])(=[O:3])[CH3:4] \gg [S:1](=[O:2])(=[O:3])[NH2:4]$$

   a. This highlights rows corresponding to the specified transformation.
5. Click on the "Pair Clusters" tab and select "Create a single cluster using the current selection."

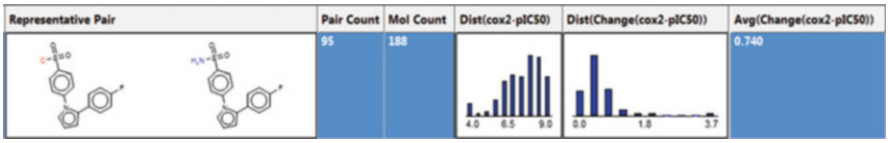


Fig. 6 Distribution plots of "cox2-pIC50" values for 188 molecules, the change in "cox2-pIC50" values for 95 pairs, and the average change in "cox2-pIC50" value for replacing CH3 with NH2

MedChem Studio then displays distribution plots for the change in value for all properties (only one property is shown here) in the spreadsheet along with other statistics such as average change in property value. An example of these distribution plots is shown in Fig. 6.

6 Building Classification and Regression Models

QSAR and quantitative structure–property relationship (QSPR) models are mathematical functions that relate molecular and atomic descriptors to biological activity or to other physical properties. Many different machine-learning algorithms can be used to create predictive models – multiple linear regression (MLR), partial least squares (PLS), artificial neural networks (ANNs), and random forest (RF), to name a few. Descriptors can be based on the atomic 3D coordinates of atoms within molecules or on the molecular connectivity alone, i.e., the 2D structure of the molecule. Our own work focuses primarily on 2D QSAR models based on artificial neural network ensembles (ANNEs) – collections of ANNs whose outputs are combined to arrive at the final result.

Regardless of the descriptors and machine-learning algorithm used, high-quality data is necessary in order to create a useful QSAR/QSPR model. One needs to thoroughly check that structures are correct and associated with the correct data value. Situations where differing experimental values exist for the same compound need to be resolved by omitting the compound, choosing a specific experimental result, or perhaps using a median or average value. It is also usually a good idea to represent the compound structure in terms of the most prevalent tautomer under the assay conditions used. Our protocol also neutralizes any ionized centers unless, like quaternary ammonium cations, they bear a permanent formal charge. Other QSAR methods may require the compounds to be in their predominant charge state at pH 7.4, e.g., a carboxylic acid would be represented as an anion. Whichever standard state is chosen, it needs to be consistently applied across the full data set as well as when generating predictions for structures not in the original data set, e.g., synthesis candidates.

The descriptors considered for inclusion in the model must be relevant to the property to be modeled. Bulk properties (logP, solubility, permeability, etc.) depend on the whole molecule. Thus, molecular descriptors can be used to accurately describe these types of properties. However, other properties such as pK_a or sites of metabolism are dependent on the atomic environment around the atom of

interest. Atomic descriptors that capture the properties of specific atoms and their environment are necessary to accurately describe these phenomena.

We built COX-1 and COX-2 inhibition ANNE models using the ADMET Modeler™ module within ADMET Predictor 6.5. A flowchart of the process used is shown in Fig. 7. It begins with computation of molecular and atomic descriptors from the 2D structure of the molecule. These descriptors are then analyzed to remove low-variant and correlated descriptors. If a descriptor has little or no variation from molecule to molecule, then it will not be able to account for changes in a molecular property or activity from molecule to molecule. If two descriptors are too highly correlated, one of them is removed.

The next step is to divide the data set into training and test sets (Dearden et al. 2009; Scior et al. 2009; Tropsha 2010). There are several methods listed in Fig. 7 to form these sets. Next, the optimal range of ANNE architectures is determined. Here, the word "architecture" indicates the level of ANN complexity. In the case of multilayer perceptron ANNs as implemented in ADMET Modeler, the complexity is governed by two parameters: the number of descriptors (ANN inputs) and the number of neurons in its hidden layer (Zupan and Gasteiger 1999). We use a standard feed forward neural network with a single hidden layer and a hyperbolic tangent activation function. The numbers of neurons and descriptors to create the best model are not known initially; thus, a range of models with varying numbers of neurons and descriptors within the prespecified ranges are created for the given training set. An "early stopping" procedure is used to prevent overtraining. Once trained, the models are used to perform predictions on the

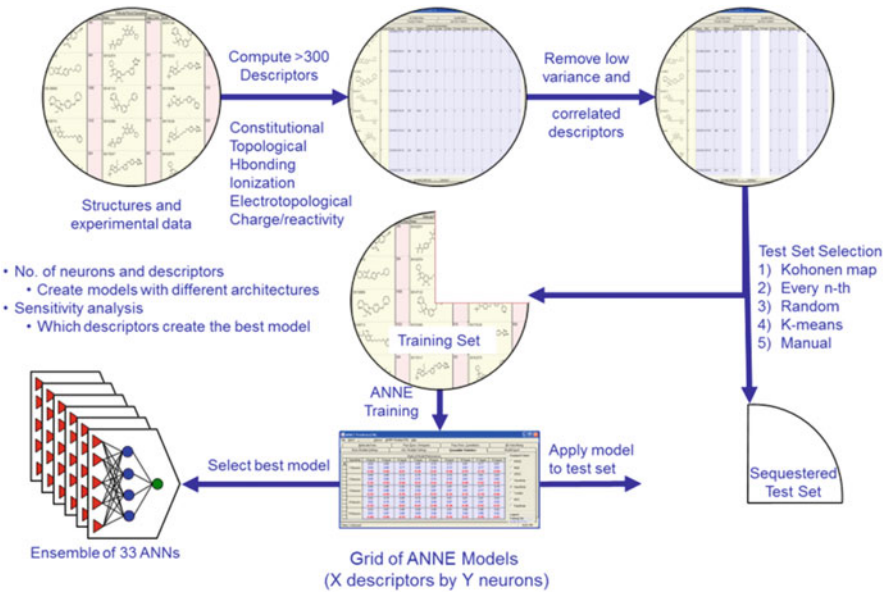


Fig. 7 General procedure for creating ANNE models

Table 1 Number of training and test compounds for each model

Model	Training pool compounds	Test set compounds	Total
COX-1 regression	172	42	214
COX-2 classification	490	54	544
COX-2 regression	384	43	427
COX-2/COX-1 selectivity	182	32	214

external test set that was withheld from the training process. Various statistics (root mean square error (RMSE), coefficient of determination, etc.) for the training and test sets are analyzed in order to select the best model. It is important that the statistics for the training and test sets are comparable in order to choose a model that is not overtrained. For example, if the training set RMSE is 0.3 and the test set RMSE is 1.0, then the model is likely to be overtrained. Once a model is selected, it can be used to predict activity or property values for new molecules.

ADMET Predictor version 6.5 was used to build a COX-1 regression model, COX-2 classification and regression models, and a COX-2/COX-1 selectivity model. The numbers of compounds in the training pools and test sets for each of the models are listed in Table 1. Compounds were categorized as positive (inhibitory) for the COX-2 classification model if their IC_{50} was 10 μM or below.

Some of the IC_{50} values were qualitative, e.g., COX-2 $IC_{50} > 10$ μM. These data were used to build the COX-2 classification model, but could not be used for the COX-2 regression model. Much of the COX-1 inhibition data was qualitative because researchers were typically looking for COX-2-selective molecules. Thus, fewer compounds were available for COX-1 models.

7 Combinatorial Elaboration and Fragment Assembly

The known COX-2-selective inhibitors in our data set contain three rings. These rings are labeled S (scaffold), A, and B in Fig. 8. The scaffold, ring S, is the most distinctive portion of cyclooxygenase inhibitors. There is some diversity in ring A, whereas ring B is always a phenyl ring with either a sulfonamide or methyl sulfone in the *para* position. We focused on generating relatively uncharacterized chemical series in our scaffold-hopping example.

Scaffold hopping was performed in two different ways – combinatorial elaboration and fragment assembly – in the design module of MedChem Studio. The former algorithm is similar to the one described by Stewart et al. (2006). Examples of transformations include reversing an amide, i.e., swapping positions of the amine and carbonyl, and replacing a carboxylic acid with a tetrazole ring. The "Combinatorial Transforms" option in MedChem Studio accesses approximately 120 potential molecular transformations developed in collaboration with medicinal chemists. Rofecoxib was used as the starting structure.

Fragment assembly generates novel molecules by replacing a designated portion of a known inhibitor with fragments from a database of drug fragments. The database of drug fragments was generated by breaking molecules from the World

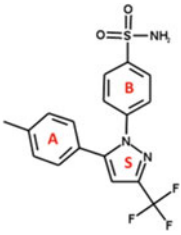


Fig. 8 Labels for the three rings in Celebrex

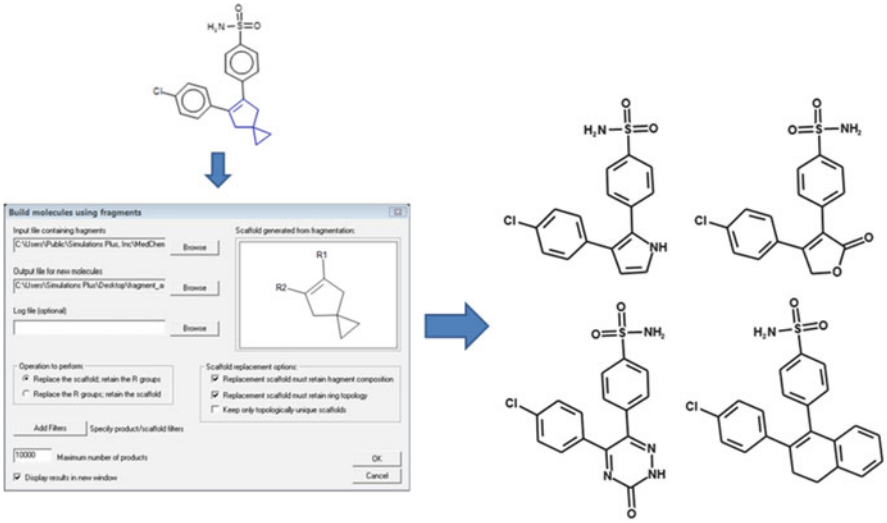


Fig. 9 Replacing a scaffold by fragment assembly. Note that an isomer of the lactone scaffold found in rofecoxib was generated

Drug Index into smaller pieces using the retrosynthetic combinatorial analysis procedure, RECAP (Lewell et al. 1998).

Again, our goal at this stage of the process was to generate novel scaffolds. Thus, we used fragment assembly to replace the scaffold in several known cyclooxygenase inhibitors. To do so, we had to:

1. Define the scaffold.
2. Specify that it is the scaffold, not the substituents, that is to be replaced.
3. Specify that the original substituent configuration and the original ring topology be retained.

In MedChem Studio, the scaffold can be defined with a search query or by holding down the left mouse button and "lassoing" the atoms in the scaffold. A few of the structures produced by applying this procedure to a known inhibitor are shown in Fig. 9.

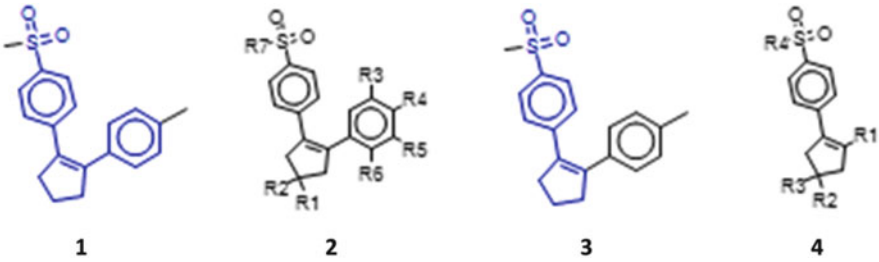


Fig. 10 Structures **1** and **2** are the representative lead and scaffold, respectively, as represented in MedChem Studio. Structures **3** and **4** are the lead and scaffold generated after redefining the scaffold

8 Virtual Library Creation by Combinatorial Enumeration

Side chains from 236 "lead" compounds – those with COX-2 $IC_{50} \leq 100$ nM – were used to create virtual libraries for each new scaffold. All included a phenyl ring with a methyl sulfone or sulfonamide in the *para* position on the B ring (Fig. 8), so these were the only two options allowed at that position. A more diverse group of potential A rings was obtained from the substituents at the corresponding position in the 236 molecules. This was achieved by clustering the compounds by ring system while specifying a minimum of three rings in the scaffold. This generated 22 clusters, including 6 singletons. The side chains for each cluster were extracted by redefining the scaffold created by MedChem Studio such that the group of interest became one of the R groups. An example for the cyclopentene-containing class is shown in Fig. 10. Structure **1** contains a very large maximum common substructure. MedChem Studio automatically generated an R table containing the seven side chain positions labeled in structure **2**. The scaffold was redefined as depicted by structure **3** to consolidate side chains R3 through R6 into the single R1 substituent shown in structure **4**. A unique set of side chains was extracted as SMILES strings and then used as input for virtual library enumeration. This resulted in 60 unique R groups. The virtual libraries were enumerated in MedChem Studio using these side chains and reactions defined as SMIRKS strings.

9 ADMET Risk™

Good biological properties are important for any drug candidate, but a good ADMET profile is also necessary. This is particularly true when an oral delivery route is desired, as is usually the case. We used ADMET Predictor's ADMET Risk™ rules to quickly screen out candidate molecules likely to have excessive ADMET liabilities. A reference subset of 2,270 orally delivered drugs from the World Drug Index were used to establish threshold values for properties computed by ADMET Predictor. The thresholds were defined so that approximately 90% of

the 2,270 compounds would pass. Large size, for example, increases the risk of poor intestinal absorption. To establish suitable size thresholds, the molecular weights, heavy atom counts, bond counts, and molecular volume were computed for the 2,270 compounds. The 90th percentile within the reference set was about 550 for molecular weight; 35 and 40 for heavy atom and bond counts, respectively; and 500 $Å^3$ for molecular volume.

We used the default ADMET Risk rules, the contributing properties for which are outlined below along with the two-letter mnemonic that is assigned to each ADMET Risk to make it easy to tell at a glance which rules were violated. As is generally the case, we focused on structures predicted to carry minimal overall risk, provided their predicted potencies were acceptable. This is a conservative approach, given that the models upon which the ADMET Risk score is based are not perfect. Then, too, risks in and of themselves may not be "show stoppers" (10% of the 2,270 compounds violate more than six of the default rules).

Absorption Risks
Sz molecular weight, number of atoms, number of bonds, and molecular volume (too big)
RB number of rotatable bonds (too flexible)
HD hydrogen bond donor count and charge (too many good H-bond donors)
HA hydrogen bond acceptor count and charge (too many good H-bond acceptors)
ch absolute charges and topological polar surface area (excessive charge)
ow S + logP, S + logD at pH 7.4, and MlogP (too lipophilic)
Pf human jejunal and MDCK permeability (low permeability)
Sw S + Sw (low water solubility)

CYP Risks
1A excessive clearance by CYP 1A2
19 excessive clearance by CYP 2C19
C9 excessive clearance by CYP 2C9
D6 excessive clearance by CYP 2D6
3A excessive clearance by CYP 3A4
Mi inhibition of midazolam metabolism by CYP 3A4
ti inhibition of testosterone metabolism by CYP 3A4

Toxicological Risks
hE inhibition of hERG
ra acute rat toxicity
Xr rat carcinogenicity in chronic feeding studies
Xm rat carcinogenicity in chronic feeding studies
Hp hepatotoxicity based on one kind of serum enzyme profile
SG hepatotoxicity based on another kind of serum enzyme profile
Mu mutagenicity in a panel of in silico Ames tests with and without metabolic activation

Pharmacokinetic Risks
fu low fraction unbound in plasma
Vd high steady-state volume of distribution

10 Physicochemical and Biological Characterization

10.1 COX-1 and COX-2 Assays

COX-1 and COX-2 IC_{50} determinations were performed by Cerep[4] using published methods (Glaser et al. 1995). The experiments measured the amount of PGE_2 formed from arachidonic acid at pH 8.0 over a 5-minute period using a recombinant enzyme expressed in transfected Sf-9 cells. For basal control measurements, arachidonic acid was omitted from the reaction mixture. The amount of PGE_2 generated was determined by homogeneous time-resolved fluorescence. Results are expressed as a percent inhibition of the control enzyme activity. Diclofenac (Fig. 11) served as the positive control in COX-1 assays and NS-398 served as the positive control in COX-2 assays.

Both standards were tested in each experiment at several concentrations to obtain an inhibition curve from which its IC_{50} value is calculated.

10.2 Thermodynamic Aqueous Solubility Assay

Thermodynamic aqueous solubility assays were also performed by Cerep using a literature protocol (Lipinski et al. 1997) that uses HPLC-MS to quantitate the amount of compound remaining in solution at room temperature after centrifugation at 2,500$\times g$ for 30 min. The dynamic range of the assay is from 1 μM to 2 mM.

10.3 Stability in Human Liver Microsomes

Human liver microsome (HLM) stability assays were performed by Absorptions Systems[5] using human liver microsomes obtained from Xenotech. Compounds were tested at a concentration of 1 μM with a preincubation time of 3 min at 37°C. Testosterone controls were run in parallel. Aliquots (100 μL) of reaction mixture were withdrawn at 0, 10, 20, 30, and 60 min, then quenched, and assayed by HPLC-MS/MS against an internal standard using electrospray ionization detection. Half-lives were estimated by fitting the percent remaining to a single-phase exponential decay curve.

[4] Eurofins Panlabs Inc. 15318 NE 95th Street, Redmond, WA 98052 USA, Tel (425) 895-8666
[5] Absorption Systems, Exton PA 19341, Tel (610) 280-7300

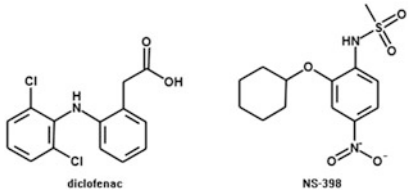


Fig. 11 Structures of positive controls used in IC_{50} determinations

10.4 LogD Measurements

The buffered octanol/water partition coefficient of our compounds was measured in 0.2 M KH_2PO_4 adjusting to pH 7.4. Testosterone (100 μM) was included in each tube as an internal control. The aqueous and octanol layers were both assayed by HPLC-MS/MS using electrospray ionization.

11 Analysis and Modeling Results

11.1 Molecular Pair Analyses

Our cyclooxygenase data set consists of 549 inhibitors – 480 from Searle and 69 from Merck. Reported COX-2 IC_{50} values ranged from 1 nM to >100 μM and COX-1 IC_{50} values range from less than 1 nM to >1,000 μM. The main structural elements include a 1,2-substituted five- or six-membered ring (see Fig. 8 for ring labels) as the scaffold. The two atoms of the scaffold connected to the A and B rings are sp^2 hybridized so this torsion angle is 0°. The sulfonyl group of the B ring (sulfonamides) is an absolute necessity for high potency.

Next, we set out to identify pairs of compounds that have very little structural difference but exhibit a large difference in COX-2 activity – a crucial step in identifying structural changes that would lead to wasting synthesis resources on compounds with little or no chance of being good COX-2 inhibitors. We specified a maximum of four bond/atom differences in the pair along with a restriction that differences occur at a maximum of two sites. Finally, the COX-2 IC_{50} values of similar molecules had to differ by at least three orders of magnitude to constitute a "cliff." This generated 143 pairs.

A few interesting activity cliffs are displayed in Fig. 12. The **5-1h/5-1d** pair in the upper-left-hand corner differs by substitution of a methyl group for a cyano group. This caused the COX-2 IC_{50} value to drop from 77.9 μM down to 3 nM! This may indicate that the nitrile group protrudes into protein resulting in a much less stable complex. A similar hypothesis can be proposed for the **1-21/1-20** pair in the lower-left-hand corner of the figure, although the methyl group of the sulfonamide would occupy a different pocket. Alternatively, removal of the methyl group may free up another hydrogen bond donor.

The two pairs on the right-hand side of the figure (**20-2/20-3a** and **21-20/21-19**) indicate that substituents on the same side of the scaffold as the phenyl sulfone are

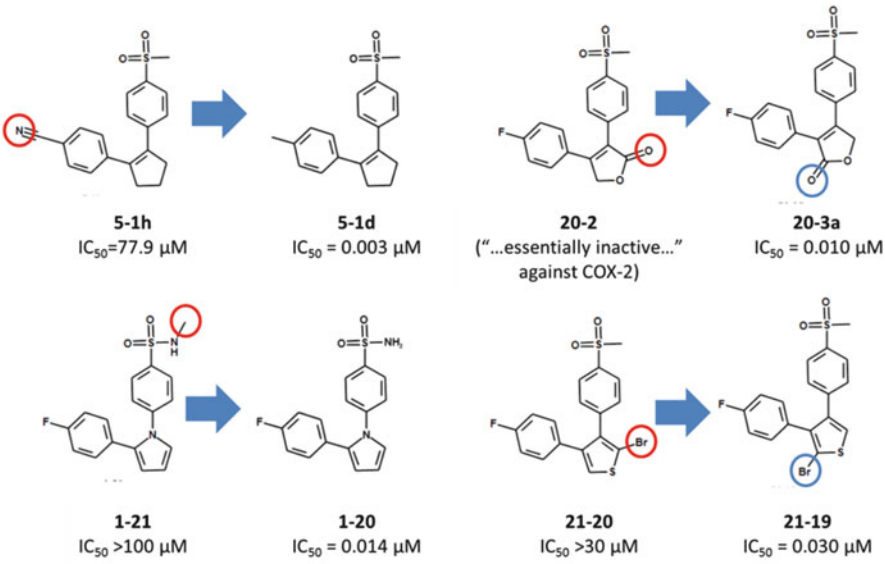


Fig. 12 Four interesting activity cliffs. COX-2 IC50 values (Chavatte, et al. 2001) are displayed below each compound's name. For compound **20-2**, the journal article reported "essentially inactive" (Prasit et al. 1999), so its IC50 was set to >100 μM. The less-active molecule is shown on the left for each pair. Structural differences between the molecules are highlighted as *red* or *blue circles*

not tolerated for the 2-furanone and thiophene scaffolds. It is important to keep these activity cliffs in mind when designing new inhibitors; however, they are dependent upon the scaffold. For example, Valdecoxib is substituted on the same side as the phenyl sulfone yet has high COX-2 potency.

The selective COX-2 inhibitors shown in Fig. 1 all bear either a methyl sulfone or a sulfonamide group at the *para* position. What are the differences in COX-1 and COX-2 activities for these functional groups across the different scaffolds in our knowledge database? Here, one can use matched molecular pair settings that correspond to small changes at single sites to gain insight into the question. Thus, the maximum number of atoms/bonds that are not a match was set to 2 and the number of sites was set to 1. This generates 586 pairs, but not all involve the methyl sulfone/sulfonamide transformation. To find pairs containing this single transformation, we used the SMIRKS string (specified in the "Reaction Query" dialog box under the "Pairs" pull-down menu) below:

$$[S:1](=[O:2])(=[O:3])[CH3:4] \gg [S:1](=[O:2])(=[O:3])[NH2:4]$$

The SMIRKS string contains an atom identifier after the colon. This creates a mapping of each atom on the left side of the "≫" to an atom on the right side. Ninety-five pairs were found. The results are displayed as distribution plots in Fig. 13. The distribution of the COX-2 pIC50 differences is either zero or positive,

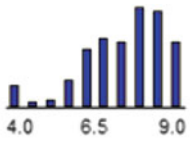
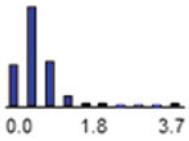
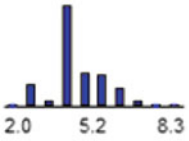
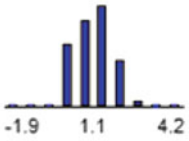
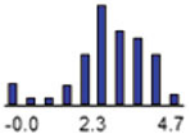
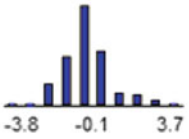
Property	pIC50 Distribution	Δ Property	Average Δ
COX-2 pIC50			0.740
COX-1 pIC50			1.067
COX-2/COX-1 selectivity			-0.327

Fig. 13 Matched molecular pair analysis for replacing methyl sulfone with a sulfonamide. Distribution plots for 188 molecules and 95 molecule pairs for COX-2 pIC50, COX-1 pIC50, and COX-2/COX-1 selectivity followed by the average change in property value are shown

indicating that the sulfonamide is always at least as potent as the methyl sulfone. A few examples of the largest effects of this structural change are shown in Fig. 14. The mean change is 0.74, indicating that – *on average* – the COX-2 IC_{50} for the sulfonamides is 5.5 times lower than for the corresponding methyl sulfone. A similar trend is observed for COX-1 activity where the average change in COX-1 pIC50 is even higher, 1.067.

This combination of effects produces an average COX-2/COX-1 selectivity (COX-2 pIC50 minus COX-1 pIC50) differential of −0.327, indicating that replacing the methyl sulfone with a sulfonamide increases COX-1 activity *more* than COX-2 activity. Thus, preferring sulfonamide analogs is in line with our goal of retaining COX-2 selectivity while increasing COX-1 potency.

11.2 QSAR Model Generation

We find that many regression models benefit from having a companion classification model to distinguish active from inactive compounds. This is because the regression model can only be trained on compounds that have quantifiable inhibitory activity, and so may not be applicable to compounds that inhibit weakly or not at all; they indicate how potent an active compound can be expected to be, but do not provide information about the much broader range of chemistries encompassed

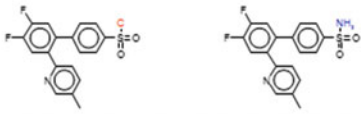
Pair	Name 1	Name 2	<Mol Ac...	<Mol Ac...	Change(...
	7-20f	7-21f	4.000	7.678	3.678
	7-16d	7-17d	4.000	7.481	3.481
	7-16e	7-17e	4.943	7.721	2.778
	7-20p	7-21p	4.281	6.481	2.200

Fig. 14 Examples of large COX-2 activity differences between methyl sulfone and sulfonamide molecule pairs

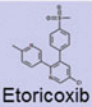
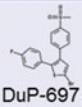
Table 2 Statistics for COX-2 classification model

Set	No. of compounds	Specificity	Sensitivity	Concordance
Training pool	490	0.83	0.88	0.87
Test	54	0.75	0.81	0.80

by inactives. Such model pairs are applied sequentially: the classification model to predict whether or not a new compound *is active*, and then – if it is predicted to be active – the regression model can be applied to determine *how active* it is likely to be.

We created a classification model for COX-2 activity for this purpose, categorizing compounds with $IC_{50} \geq 10$ μM as inactive and compounds with $IC_{50} < 10$ μM as active. The data set was divided into a training pool of 490 molecules and a held-out external test set of 54 compounds. Both the training pool and test set contain 78% actives. The model used 3 neurons and 47 descriptors. The specificity, sensitivity, and concordance of the model are shown in Table 2.

The classification model results for marketed COX-2 selective inhibitors and a few other molecules are shown in Fig. 15. All of the marketed compounds are predicted to inhibit COX-2 with fairly high confidence except for rofecoxib, where the confidence is 68%. Correct prediction of the COX-2 activity of furan derivatives proved less reliable: the model correctly predicts the COX-2 inactivity of **20-2**, but the isomeric **20-3a** is a false negative, i.e., it is predicted not to inhibit COX-2, but experimentally it is a fairly potent inhibitor with a COX-2 IC_{50} of 10 nM. Such regiochemical subtleties can be difficult for 2D molecular descriptors to capture.

Molecule	Observed COX-2 Inhibitor?	Predicted COX-2 Inhibitor?
 Celecoxib	Yes	Yes(97%)
 Valdecoxib	Yes	Yes(89%)
 Etoricoxib	Yes	Yes(97%)
 DuP-697	Yes	Yes(92%)

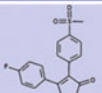
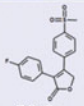
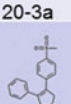
Molecule	Observed COX-2 Inhibitor?	Predicted COX-2 Inhibitor?
 20-2	No	No(59%)
 20-3a	Yes False negative	No(60%)
 Rofecoxib	Yes	Yes(68%)

Fig. 15 COX-2 classification results for marketed COX-2-selective inhibitors and other compounds. The numbers in parentheses are the confidence estimates

We planned on only synthesizing a small number of compounds, so we were willing to accept a false negative. False-positive predictions are much more costly because the compound could be selected for synthesis and result in an inactive compound.

Plots of observed versus predicted values for COX-2 pIC50, COX-1 pIC50, and COX-1/COX-2 selectivity are shown in Fig. 16. Table 3 shows statistics for each regression model.

12 Scaffold Hopping

Rofecoxib was used as the starting structure for combinatorial transformations using the default settings in MedChem Studio. Forty-six new analogs were generated. Of these, 11 were missing the phenylsulfonyl group and were discarded as unlikely to be of interest. Thirteen of the remaining 35 compounds were modified in the phenyl ring or had a methylene group inserted between the scaffold and one of the side chain rings. These compounds and three additional products where the scaffold was opened were also eliminated from further consideration.

The thiolactones shown in Fig. 17 were chosen as interesting scaffolds to pursue. Our metabolic models indicated that **Scaffold 1** is at risk of getting hydroxylated at carbon number 5. Adding a methyl group in this position to block oxidation yields **Scaffold 2. Scaffold 3** features a spirocyclopropyl group at C 5, which should completely block metabolism.

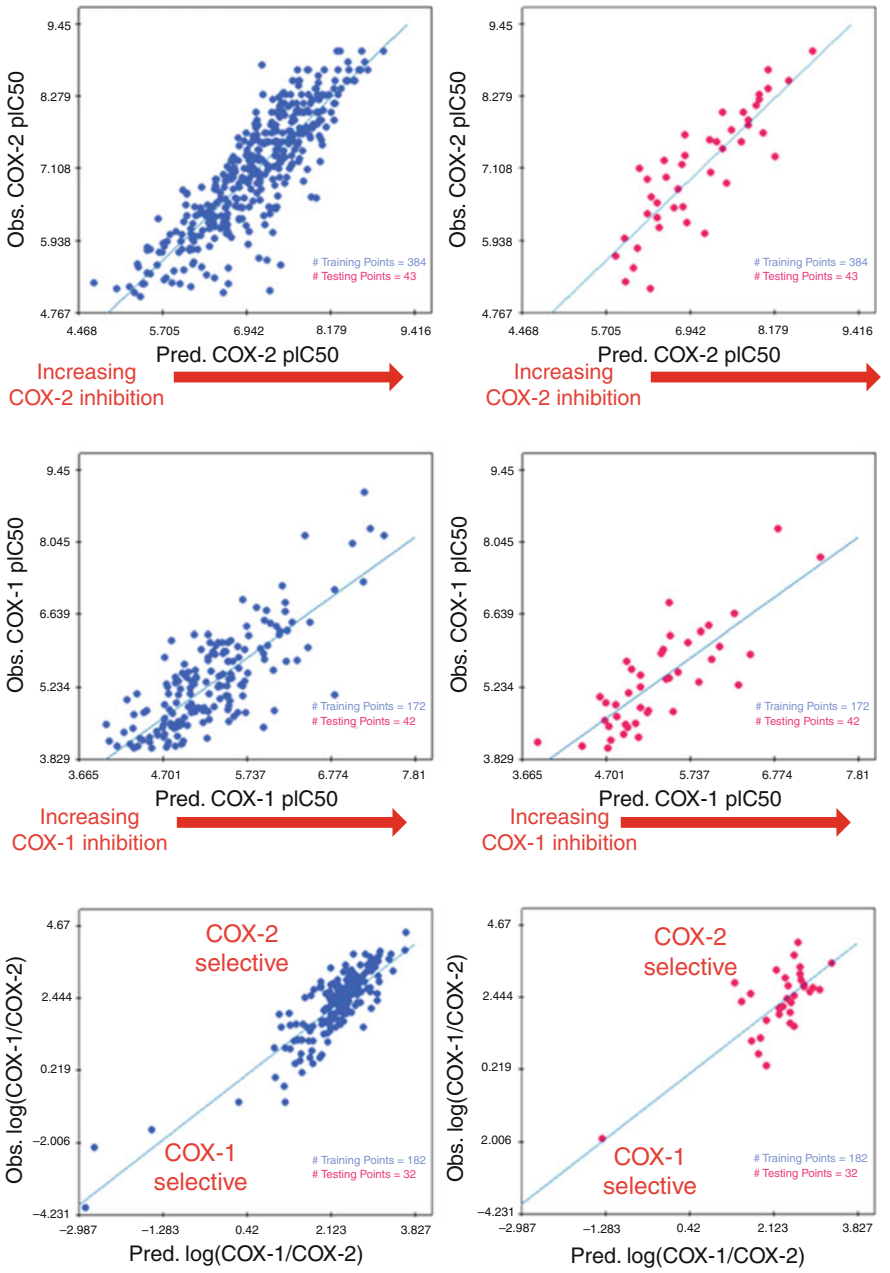


Fig. 16 Observed versus predicted plots for (*top*) COX-2 pIC50, (*middle*) COX-1 pIC50, and (*bottom*) log(COX-1/COX-2) activity. The plots on the left are for the training pool and the ones on the right are for the external test set

Table 3 Statistics for regression models

Model	Set	No. of compounds	RMSE	R^2	Slope	Intercept
COX-2	Train	384	0.493	0.746	1.070	−0.474
	Test	43	0.485	0.741	1.090	−0.614
COX-1	Train	172	0.571	0.652	1.140	−0.755
	Test	42	0.561	0.662	1.150	−0.829
Selectivity	Train	182	0.543	0.792	1.200	−0.440
	Test	32	0.746	0.567	1.080	−0.176

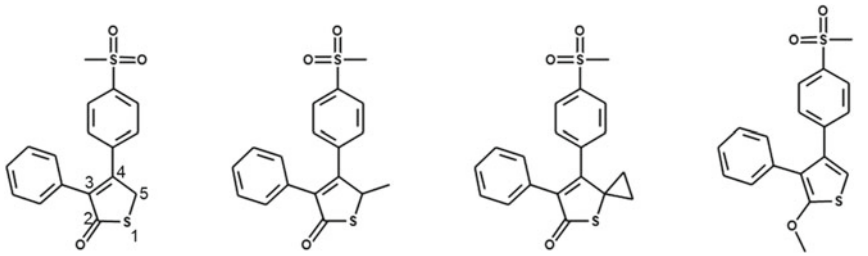
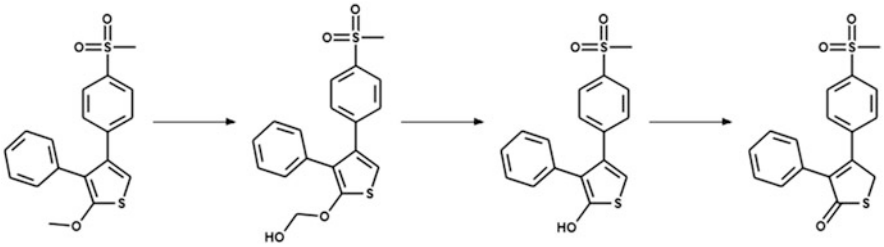


Fig. 17 Scaffold ideas based on combinatorial elaboration and metabolic considerations

Scaffold 4 is intended to be a prodrug predicted to be demethylated by human cytochromes to generate the corresponding hydroxy thiophene, which will in turn tautomerize to **Scaffold 1**. The overall process is:



A few of the most potent COX-2 inhibitors were used as templates for "fragment assembly." The ring topologies of commercial COX-2 inhibitor scaffolds are similar, so many of the scaffolds generated from different initial structures were identical. The lactone ring of rofecoxib, the pyridine ring of valdecoxib, and several of the scaffolds from known COX-2 inhibitors were generated with this procedure. Scaffolds where *both* connection atoms that attach the two side chains were *not* sp^2 hybridized were eliminated. Figure 18 shows five scaffold ideas that were generated by fragment assembly and advanced to the next stage of the design process.

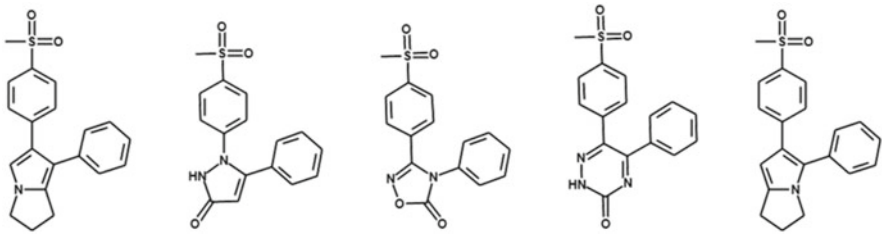


Fig. 18 Scaffold ideas generated by fragment assembly

13 Combinatorial Library Generation

Each of the nine scaffolds was decorated with 60 "A" rings and 2 "B" rings. The scaffolds are unsymmetrical so *two* 2×60 sub-libraries were created for each scaffold in order to cover more chemistry space. This is illustrated for **Scaffold 7** in Fig. 19. Thus, the nine virtual libraries each contained 240 compounds.

14 Candidate Selection

We used our cyclooxygenase classification and regression models to predict COX-1 and COX-2 activity of the virtual compounds, and we predicted their ADMET Risk scores. Distribution plots of each of these predicted properties for the various virtual libraries are shown in Fig. 20. The COX-2 classification model predicts that most of the thiolactone molecules will inhibit COX-2. Most of the scaffolds obtained from fragment assembly lead to compounds predicted to inhibit COX-2, except for compounds bearing **Scaffold 8**.

The predicted COX-2 pIC50 values are above 7 ($IC_{50} < 100$ nM) for **Scaffolds 1–6** and **9. Scaffold 7** has fewer compounds predicted to have a COX-2 pIC50 above 7 compared to these libraries. The COX-2 pIC50 values for **Scaffold 8** are typically 7 or less, consistent with the classification model. The predicted COX-1 pIC50 values are considerably lower than the COX-2 pIC50 values. **Scaffold 5** has the highest predicted COX-1 activity, although **Scaffolds 1–3** also contain fairly potent COX-1 inhibitors.

ADMET Risk is greater than 6 for about 10% of the focused WDI subset. Thus, a value of 7 or more indicates significant barriers to drug development. Only 7 of the 2,160 virtual compounds had an ADMET Risk of 7 or more. Thus, overall, the molecules have good predicted ADMET properties. **Scaffold 4** had the least favorable ADMET Risk distribution, whereas **Scaffold 6** had the most favorable distribution.

The next step in the process involved filtering the virtual libraries for "out-of-scope" predictions and adequate COX-1 and COX-2 potency. In ADMET

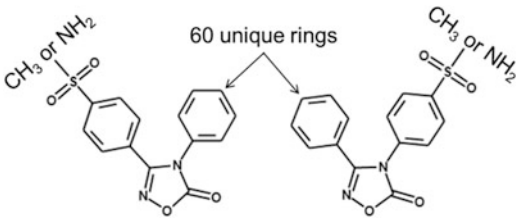


Fig. 19 Scaffold 7 sub-libraries

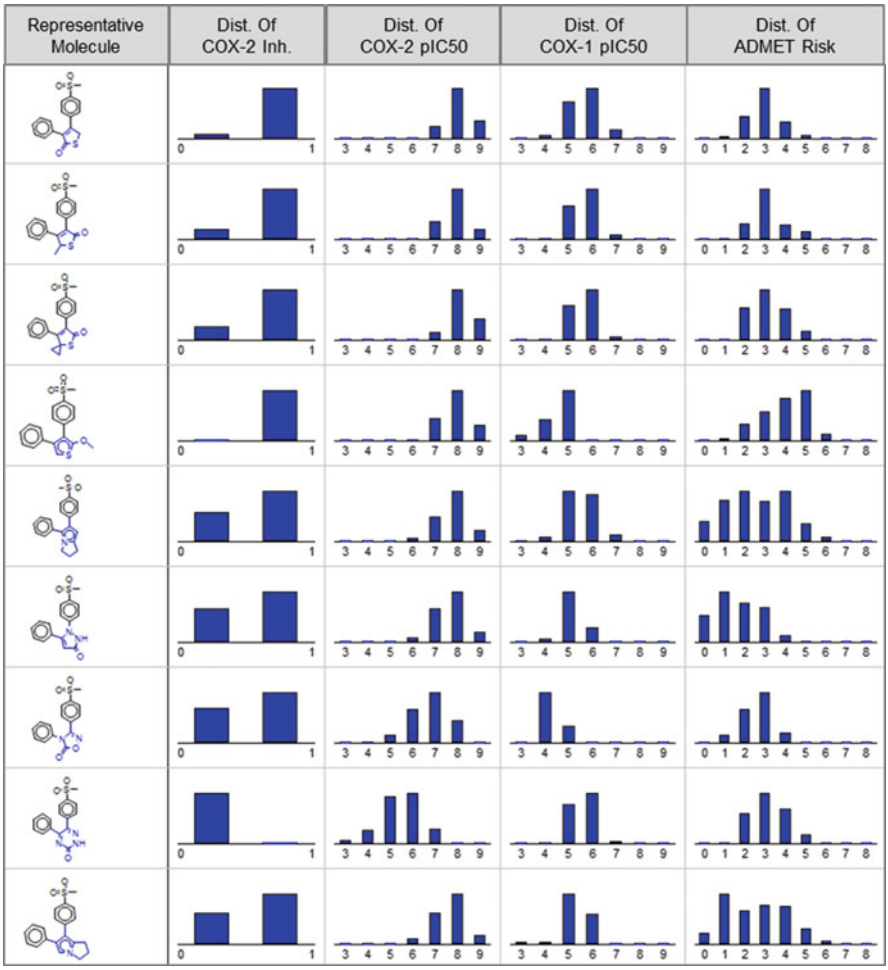


Fig. 20 Distributions plots for each virtual library. "COX-2 Inh." is the COX-2 classification model, 0 indicates inactives, and 1 indicates actives. COX-2 pIC50 and COX-1 pIC50 are from the COX-2 and COX-1 regression models, respectively

Table 4 Compound filtering criteria and number of compounds remaining after each stage

Criteria	Removed	Remaining
Remove OOS COX-2 classification model	52	2,108
Remove COX-2 classification = No	700	1,408
Remove OOS COX-1 regression model	187	1,221
Remove OOS COX-2 regression model	40	979
Remove COX-1 predicted $\leq$1 μM	248	933
Remove COX-1/COX-2 > 100	691	242

OOS stands for "outside of scope" of the model

Predictor, a model's applicability domain is defined as the minimum and maximum (plus a 10% tolerance) descriptor values for the training pool compounds. Prediction on compounds where one of the descriptor's values is outside of this domain is termed "out-of-scope." Table 4 shows the various filtering criteria and the number of compounds remaining after applying each filter in the sequence.

We first removed 58 compounds that were outside the scope of the COX-2 classification model. Removal of compounds predicted to be non-inhibitors of COX-2 was followed by removal of the 267 compounds that fell outside the scope of the COX-1 or COX-2 regression models. Next, compounds with COX-1 IC_{50} predicted to be less than 1 μM were removed, followed by removal of compounds predicted to have a COX-1/COX-2 IC_{50} ratio greater than 100. Overall, 242 compounds were excluded under our filtering criteria.

At this point, we needed to reduce the number of candidates to around 12 compounds to present to our internal review committee. We rejected compounds containing bicyclic side chains because they might be too bulky. We also rejected side chains that might cause toxicity (e.g., thiophene) or were deemed less stable.

In the end, we settled on eight candidate structures for which we solicited synthesis quotes from various contract chemistry organizations (Fig. 21). Four candidates contained a thiolactone and four shared a dihydropyrrolizine scaffold. All of the proposed compounds also contain a sulfonamide as opposed to a methyl sulfone since the MMPA indicted that sulfonamides are typically more potent for both COX-1 and COX-2. In each case, a "flipped" version in which the A and B rings were interchanged was included along with the candidate generated by the approach described above. This decision was made based on the activity cliff analysis discussed above.

No ADMET Risks were predicted for the dihydropyrrolizines. The thiolactone-containing molecules each exhibit ADMET Risks for low solubility and for low fraction unbound in plasma. The default threshold for solubility risk was 5 μg/mL, and the thiolactone-containing molecules had predicted solubilities ranging from 1 to 2 μg/mL. The predicted fraction unbound in plasma for these compounds was slightly below the ADMET Risk threshold of 0.035. These ADMET properties are shared by the marketed COX-2-selective inhibitors.

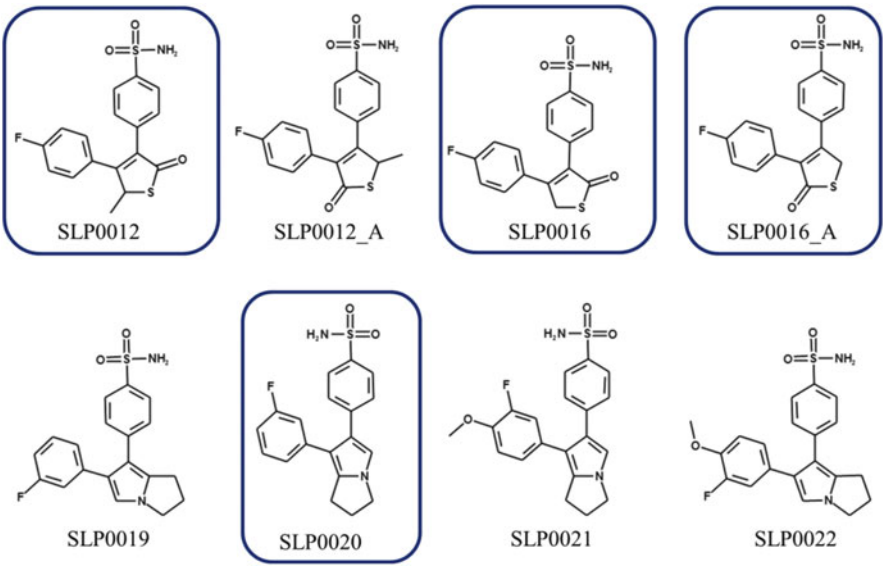


Fig. 21 Compounds sent to contract research organizations for synthesis quotes. The four compounds outlined in *blue* were synthesized

15 How Good Were Our Predictions?

Predicted and experimental properties for the four compounds that were synthesized are shown in Table 5. Attempts to synthesize the other four were unsuccessful. The experimental and predicted logP values are in good agreement. The predicted solubilities are also in good agreement with the experimental values – within the factor of about threefold associated with most of our regression models. The largest discrepancy is for **SLP0016_A**, where our model predicted a solubility of 7 μM, which was below the experimental value of 24 μM, although it is almost within a factor of 3. Note, however, that the solubility reported for **SLP0016_A** is much higher than that of its isomer and the other thiolactone. It seems possible that the experimental result is not correct.

SLP0016_A is the most resistant of the four compounds to CYP metabolism by human liver microsomes (HLMs). Seventy-one percent of the compound remained after incubating the compound in human liver microsomes for 60 min, i.e., the half-life was longer than the duration of the experiment. **SLP0020** exhibited the highest experimental intrinsic clearance (CL_{int}) of 93 μL/min/mg microsomal protein. Our CYP metabolism models predict that the *para* position on the 3-fluorophenyl group would be hydroxylated. Moving the fluorine from the 3 position to the *para* position in **SLP0020** is predicted to block this site of metabolism and lower intrinsic clearance.

Table 5 Experimental and predicted properties

Property	SLP0012	SLP0016	SLP0016_A	SLP0020
logP	2.2	2.0	2.1	3.6
Predicted logP	2.9	2.5	2.6	3.5
Solubility [μM]	5.8 ± 0.56	2.0 ± 0.36	23.6 ± 0.57	9.0 ± 1.00
Predicted solubility [μM]	5.1	6.6	7.1	20.9
HLM CL_{int} [μL/min/mg protein]	53	37	12	93
Predicted CL_{int} [μL/min/mg protein]	27	21	21	29
Plasma stability (% remaining after 2 h)	5	43	80	99
COX-1 IC_{50} [μM]	23.0	0.51	0.14	0.77
Predicted COX-1 IC_{50} [μM]	2.32	1.763	0.617	5.601
COX-2 IC_{50} [μM]	11.0	2.10	0.26	0.13
Predicted COX-2 IC_{50} [μM]	0.012	0.017	0.054	0.026

The predicted CL_{int} values are obtained by summing the individual CL_{int} predictions for CYP1A2, 2C9, 2C19, 2D6, and 3A4

Unfortunately, the thiolactones turn out to be unstable in human plasma, with only 5% of **SLP0012** remaining after a 2 h incubation. We suspect that is due to unanticipated hydrolysis by the plasma enzyme paraoxonase, whose natural function is to hydrolyze homocysteine thiolactone in the circulation (Yilmaz 2012). Given that our thiolactone rings are unsaturated and lack the amino substituent of the natural substrate, this came as an unpleasant surprise. It bears noting, however, that such a lack of cooperation from biology is not uncommon in drug discovery. As expected for an enzymatic process, the stability in plasma was dependent on structure – with the **SLP0016_A** analog, 80% remained after a 2 h incubation in plasma.

The compounds also had much more COX-1 potency than was predicted by the COX-1 IC_{50} model. On the other hand, the COX-2 IC_{50} model predicted more COX-2 activity than was experimentally observed. Nevertheless, we were very satisfied with the activity of the compounds we had designed.

SLP0016 and **SLP0016_A** differ in the position of their carbonyl oxygen. We predicted that **SLP0016_A** would be more potent against COX-1 than **SLP0016**, which was experimentally observed. On the other hand, our COX-2 IC_{50} model predicted higher COX-2 potency for **SLP0016** than for **SLP0016_A**, whereas in fact **SLP0016_A** was eight times as potent as **SLP0016**. This fit with the general trend uncovered for lactones in our activity cliff analysis, which is what led us to include **SLP0016_A** in our list of candidates. It would have been interesting to see if the trend would have held true for **SLP0012** and **SLP0012_A** as well, but the latter compound resisted our contractor's synthetic efforts.

SLP0020 has a cyclooxygenase inhibition profile close to our goal. It is selective for COX-2 over COX-1, though we would have preferred more COX-2 activity and less COX-1 activity than what was experimentally observed. We believe that only a relatively small amount of optimization of **SLP0020** would be required to increase

the COX-1 IC_{50} value to >1 μM and decrease the COX-2 IC_{50} value below 100 nM, but that would have gone beyond the scope of our proof-of-concept project.

16 Conclusion

This chapter uses our cyclooxygenase inhibitor design project to illustrate how cheminformatics tools typically employed in drug discovery are applied in practice. Carrying the design process through synthesis and testing provided useful insight into the degree of agreement one can expect between experimental results and predictions. Data from several journal articles was used to create a knowledge database, which was then analyzed by identifying activity cliffs and performing MMPA. One particularly striking set of activity cliffs prompted us to include several isomeric pairs in our list of synthetic targets, one of which (**SLP0016_A** and **SLP0016**) was successfully synthesized. MMPA led us to target one specific structural class (sulfonamides) for synthesis as well as a potential backup class (sulfones).

A classification model was created to differentiate potent inhibitors from those that were less potent, and regression models were created so we could assess target potency and isozyme selectivity. Several scaffold ideas were generated using combinatorial transformations and fragment assembly. Virtual libraries were created from those scaffolds and side chains associated with active compounds. The virtual libraries were then filtered based on their predicted activity and selectivity along with their ADMET Risk values. The latter filtering eliminated compounds with less desirable predicted ADMET properties.

We were ultimately able to synthesize and test four of our eight candidates. SLP0012 was very weakly active against both COX-1 and COX-2. The IC_{50} values of the rest of the molecules varied from 130 nM (SLP0020 COX-2 IC_{50} value) to 2 μM (SLP0016 COX-1 IC_{50} value). The plasma and microsomal stability pose significant challenges to developing these classes of inhibitor as drugs, but we believe that they represent good leads for subsequent optimization of cyclooxygenase potency and selectivity.

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Implementation and Use of State-of-the-Art, Cell-Based In Vitro Assays

Gernot Langer

Contents

Abstract

The impressive advances in the generation and interpretation of functional omics data have greatly contributed to a better understanding of the (patho-)physiology of many biological systems and led to a massive increase in the number of specific targets and phenotypes to investigate in both basic and applied research. The obvious complexity revealed by these studies represents a major challenge to the research community and asks for improved target characterisation strategies with the help of reliable, high-quality assays. Thus, the use of living cells has become an integral part of many research activities because the cellular context more closely represents target-specific interrelations and activity patterns. Although still predominant, the use of traditional two-dimensional (2D) monolayer cell culture models has been gradually complemented by studies based on three-dimensional (3D) spheroid (Sutherland 1988) and other 3D tissue culture systems (Santos et al. 2012; Matsusaki et al. 2014) in an attempt to employ model systems

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more closely representing the microenvironment of cells in the body. Hence, quite a variety of state-of-the-art cell culture models are available for the generation of novel chemical probes or the identification of starting points for drug development in translational research and pharma drug discovery. In order to cope with these information-rich formats and their increasing technical complexity, cell-based assay development has become a scientific research topic in its own right and is used to ensure the provision of significant, reliable and high-quality data outlasting any discussions related to the current "irreproducibility epidemic" (Dolgin 2014; Prinz et al. 2011; Schatz 2014). At the same time the use of cells in microplate assay formats has become state of the art and greatly facilitates rigorous cell-based assay development by providing the researcher with the opportunity to address the multitude of factors affecting the actual assay results in a systematic fashion and a timely manner. This microplate-based assay development strategy should result in the setting up of more robust and reliable test systems that ensure and increase the confidence in the statistical significance of the actual data generated. And, although assay miniaturisation is essential in order to achieve this, most, if not all, cell-based assays can be easily reformatted and adapted to be used in this format in a straightforward manner. This synopsis aims at summarising valuable, general observations made when implementing a diverse set of functional cellular in vitro assays at Bayer Pharma AG without claiming to deeply review all of the literature available in each and every detail. In addition, phenotypic assays (Moffat et al. 2014) or label-free detection methods (Minor 2008) are not discussed. Although this essay tries to cover the most relevant technological developments in the field, it nevertheless may express personal preferences and peculiarities of the author's approach to state-of-the-art cell-based assay development. For additional reviews covering the actual field, see Wunder et al. (2008) and Michelini et al. (2010).

Keywords
Assay/instrumentation/methods · Assay/methods · Cell culture techniques · Cells/drug effects · Chemical probes · Cryopreservation · Data interpretation, statistical · Drug discovery/methods · Miniaturization/methods · Reproducibility of results

1 Starting with a Short Historical Perspective[1]

In vitro cell-based research originated about a century ago with the first attempts to culture 3D animal tissues ex vivo, and it took roughly 50 years to gradually establish all the basic principles and most of the fundamental routines for the isolation and cultivation of cells still valid and highly important today. The generic nature and applicability of these techniques not only fostered the generation of

[1] For a more detailed description, see Freshney (2000a).

highly valuable and reproducible results but also led to continuous rise in both basic and applied cell-based research activities. The 1960s experienced an accelerated increase and rapid diversification in the field based on major achievements in the culturing of cells with finite life span, the development of tumour-derived immortalised cell lines as well as rapid progress in mass cell production and storage. A decade later, the introduction of cell fusion and recombinant DNA technologies along with the widespread use of the first high-density, cell culture and assay formats[2] firmly established the central role of cell-based research and in vitro assays in both academia and industry.[3] As a major consequence of this development, the 1980s and 1990s experienced almost exponentially growing research activities relying on cell-based, in vitro assays. Because of this more and more complex scientific and challenging numeric throughput, goals came into reach, generating the need to further miniaturise and automate the cell-culturing methods and cell-based assay techniques as well as the demand for intensified interdisciplinary scientific exchange. This trend became the major driving force for the implementation of high-throughput experimentation and screening approaches in pharmaceutical drug research[4] and academic probe development.[5] Most of the underlying concepts and strategies, workflows and core-technology platforms reached maturity shortly after the turn of the millennium. By now, their impact is widely proven and accepted with cell-based in vitro assays comprising roughly half of the screening and research activities – something that would not have been possible without employing high-density plate microplate formats (i.e. 96, 384 and 1536 well).

2 Scaling Down to Optimally Implement Robust and Reliable Test Systems

However, miniaturisation in cell-based assay development isn't primarily about increasing the number of assay points per se or cost reduction due to lower reagent consumption, although both aspects do represent major benefits and driving forces (see Table 1). In fact, opting for a miniaturised assay protocol in the first place

[2] That is, the 96-well plate described by Gyula Takátsy in 1951 already

[3] Aside from the growing public debate over the disproportionate use of experimental animals in certain research areas

[4] See: Foundation of the Society for Biomolecular Sciences (SBS) in 1994 (http://www.slas.org/about/who-we-are/society-for-biomolecular-sciences/) now an integral part of the Society for Laboratory Automation and Screening (SLAS) established in 2010 (https://www.slas.org/).

[5] As exemplified by the foundation of (a) the NIH Chemical Genomic Center (NCGC) in 2004, now a part of the National Center For Advancing Translational Science (NCATS; established 2012; http://www.ncats.nih.gov/research/reengineering/ncgc/ncgc.html) or (b) the Innovative Medicines Initiative (IMI; http://www.imi.europa.eu/content/home) established in 2008 as well as (c) the establishment of the National Cancer Institute Chemical Genomics Consortium in 2009 (http://dctd.cancer.gov/CurrentResearch/cbc/20090810_meeting.htm)

Table 1 Cell culture media requirements and costs of consumables in standard cell culture vessels compared to higher-density microplates

Dimensions			Cell culture media requirements (fill quantity)									Productivity	Costs (consumables)	
		Surface area	µL per well			mL per plate			mL/void volume corrected (+20%)			Fold increase in datapoint numbers (reference: 6-well format)	Plates** (per plate)	Tips*** (per tip)
Well number	Layout	Average (mm^2)	Optimal	Maximal From	Maximal To	Optimal	Maximal From	Maximal To	Optimal	Maximal From	Maximal To			
6	2 × 3	962	1,924	2,000	5,000	5.77	12.00	30.00	6.93	14.40	36.00	1	1.99 €	−/−
24	4 × 6	194	388	500	3,000	4.66	12.00	72.00	5.59	14.40	86.40	4	2.33 €	−/−
96	8 × 12	34	68	100	300	3.26	9.60	28.80	3.92	11.52	34.56	16	3.40 €	0.09 €
384	16 × 24	9	18	30	100	3.46	11.52	38.40	4.15	13.82	46.08	64	6.90 €	0.11 €
1536	32 × 48	2	4	4	10	3.07	6.14	15.36	3.69	7.37	18.43	256	30.40 €	−−/−−

Going for high-density formats (i.e. ≥ 96 well) should result in a reduction of media consumption by about 20–30%. In addition, cell culture media requirements do not increase even when going for still higher-density formats, i.e. >384-well plates. On the other hand, the numbers of tests being performed (productivity) increases at least 16-fold. Optimal* is adding cell culture media according to generally accepted standards for optimum gas exchange (fill level = 2 mm). Plates** are price per plate, when ordering 1,000 plates of a standard, high-quality, sterile cell culture plate with lid (for use in assay development). Please note, however, that once established, most cell-based assays do not need to be performed in sterile cell culture plates! Tips*** are list price per tip when ordering standard, high-quality, nonsterile 50/125 µL tips predominantly used in the 96-well/384-well experiments. (a) −/− not given: only the tip prices for use in high-density plate formats are listed. (b) −−/−− not given: 1536-well format pipetting usually requires the use of multichannel dispensers, although tips used in 384-well formats can be used

means implementing new concepts, strategies and workflows in order to address today's most urgent challenge: to deliver information-rich, high-quality scientific data in rapid succession in order to endure in a competitive research environment while also guaranteeing robust and reliable results. This especially holds true, when using an in vitro cell-based assay system. Implementing state-of-the-art microplate formats and assay miniaturisation allows for a much better identification of potential assay weaknesses and gives the researcher the opportunity to carefully address the "way too many" individual and interrelated effects of parameters affecting the final assay results (by permitting to systematically address these topics upfront and in a timely manner). Thus, taking the scientific challenge and transforming low-throughput benchtop formats into miniaturised, microtitre plate assays will – in most of the cases – result in the setting up of a more robust and reliable test systems, which in conjunction with the appropriate controls ensure and increase the confidence in the statistical significance of the actual data generated, something almost per se justifying the initial, quite high investments in the essential labware, i.e. electronic multichannel pipettes and dispensers as well as microplate-format compatible readers.

Once implemented, however, the increasing costs for consumables have to and can usually be balanced with the significantly reduced, actual costs per well. And although a continuous increase in the amount of datapoints generated has to be expected and taken seriously from the cost perspective, the value of high-quality data readily obtained from miniaturised, robust cell-based assay systems certainly warrants heading into this direction.

3 Working with Cells in a Miniaturised Format

Whenever using cells cultured for microplate-based assays in vitro, it is of utmost importance to keep in mind that both routine cell culture and assay (culture) workflows considerably influence the final microplate assay results. Thus, one is well advised to at least double-check the net effects of even subtle changes in both of these two processes and revise them if necessary – starting from the existing protocols of course.

4 Routine Cell Culture

Aside from special cases, where cells have to be grown and tested under non-proliferative conditions, actively proliferating cells are considered the most appropriate source for assaying. However, each cell line has a different seeding density, plating efficiency and doubling time. As this specific growth behaviour usually doesn't fit the regular working day, the experimenter quite often finds himself/herself in a cell supply bottleneck situation when it comes to having to grow and harvest a certain number of cells in time for assaying – making cell provision a limiting factor both with regard to quality and throughput. Thus, when it comes to routine subculturing, it may be helpful to evaluate whether splitting the

cells using twofold, i.e. 1:2, 1:4 and 1:8, media dilutions instead of the "usual" 1:5 and 1:10 splitting routines results in a workflow that is in synchrony with the doubling interval[6] of the individual cell line used and may contribute to improved assay results. Although this still doesn't prevent the experimenter from sometimes ending up at different cell densities at harvest, it may improve the chances of harvesting (enough) cells in their exponential growth phase. When for example harvesting 7,000,000 exponentially growing cells in sub-confluent state from a 162 cm^2 flask chances are high to also assume that every cell count $>$ 2,500,000 cells harvested from less confluent cultures should primarily consist of exponentially growing cells and be (working) fine. Any cell count below and above these arbitrarily chosen lower and upper margins should be considered inappropriate for further processing and experimentation. In addition, to ensure proper and reproducible functional assay responses of a cellular system, it is very important – and very easily done – to always record and document the cell numbers obtained when subculturing cells on a regular basis. If undetected, any deviation can seriously affect experimental results. Furthermore, this simple procedure also detects any drift in cell line activity due to ageing processes and/or contamination as added value.

5 Cells as Reagents (Frozen Cells)

The use of frozen cells[7] can be very helpful to free the lab from the uncertainties of continuously ongoing routine cell culture. Although this has become a mainstay in the screening campaigns of many pharma companies (Cawkill and Eaglestone 2007; Zaman et al. 2007), surprisingly little detailed information regarding the actual preparation and resuscitation of cells to be used as reagents has been published. Today, the vast majority of the cell-based assays developed in our department are making use of this straightforward, highly robust and reproducible approach providing the experimenter with almost the same experimental freedom that a biochemical test system brings about in addition to delivering high-quality, functional assay data. In fact, what started years ago as a measure to primarily free the experimenter from routine cell culture efforts and assay cell provision (Bergsdorf et al. 2008) and with rather simple screening assay designs has developed into a veritable success story today involving bulk frozen cell provision by CROs and an almost 100% routine use of frozen cells in complex functional

[6] See Freshney (2000b).

[7] The term frozen cell describes the use of freshly resuscitated cells shortly after their recovery from liquid nitrogen storage and should not to be mistaken for the use of division-arrested cells, as exemplified/described by Digan et al. (2005).

screens[8] and cell-based follow-up activities[9] (Schulze et al. 2015). However, the initial production of frozen cells for assay development and assay validation is still done in-house and on a small lab scale – something that can easily be recapitulated in an academic setting as well – based on scientifically sound, technical solutions that combine existing knowledge on the field of cryopreservation and resuscitation of cells while keeping the conditions of the functional assay to be performed in mind. In a way this asks for challenging the status quo of existing cell-based assay procedures. However, in the course of establishing quite a large number of frozen cell-based functional assays, the following generic approach was found to be quite helpful:

Most frozen cell assays can be implemented using standard cryopreservation procedures (Stacey and Masters 2008). Keeping the assay readiness of the frozen cells in mind, one is well advised to also evaluate potential positive effects resulting from FBS replacement by charcoal-treated serum. In addition, the feasibility of starvation techniques or the immediate use of appropriate assay media for cryopreservation should be tested.

With regard to the resuscitation of cells, great care has to be taken not to mechanically stress damage or disrupt the cells while defrosting, which generally means to start by rapidly thawing the vials at 37°C. Immediately afterwards, fast, albeit careful transfer of the cells is mandatory by decanting the vial contents into 15 mL of prewarmed medium to dilute out any DMSO. Gently resuspending the cells by swirling is used to assist this process. Then the cells are harvested via centrifugation using very low g-numbers and short centrifugation times even though this might result in the loss of a very few percent of cells. Here the aim is not to end up with a densely compact cell pellet, but to obtain quite a loosened cell pellet instead, which allows for immediate generation of a monodisperse single-cell suspension at the very first contact with new, prewarmed medium. A second washing step following the exact same procedure should be considered in case of highly DMSO-sensitive cells. Although the general impression is that the use of full growth medium – without any antibiotics – for resuscitation is utterly sufficient, the use of alternative conditions should also be checked.[10] Next, gentle decanting is used again to get rid of all washing media.

In our hands the actual assay readiness of frozen cells is achieved in a second step. Assay performance can be greatly improved by keeping the resuscitated cells as monodisperse suspension in a specific recovery medium for a certain amount of time prior to their actual use – in analogy to the actual lag phase necessary for the cells to attach to the cell culture plate and start growing. Whereas most of the cells can be used virtually at once, the influence of a 1–2 h recovery time on the final assay read-out should always be checked for improvement of assay results. This

[8] Demanding for the supply of 1–5 × 10^8 cells per screening day

[9] From routine pharmacological profiling on a weekly basis to approaches based on frozen cells transiently transfected with mutant receptors to test hypotheses from computational chemistry

[10] That is, a medium version based on charcoal-treated serum, medium only or even just PBS

also helps to address any potential influences of sometimes inevitable "prolonged cell handling times" due to the time it takes to prepare a larger number of plates. Sometimes we also observed that recovering the cells overnight while shaking them in an Erlenmeyer flask greatly improved assay performance. In doing so a fully synthetic medium was used, which prevents cell aggregation and attachment (data not shown). Whereas normal recovery procedures require gentle swirling of the cells from time to time only to keep them in suspension, prolonged incubation times may demand the cells to be resuspended at lower densities and more rigorous shaking to prevent cell clotting, nutrient starvation or pH changes as well as oxygen deprivation. Again, different recovery media should be tested always keeping the final assay conditions in mind, i.e. replacement of full medium by media with reduced serum levels or charcoal-treated serum, medium without serum or even PBS only. In addition, the actual recovery temperature used should be checked – although we've rarely observed the necessity of having to incubate the cells at 37°C. In cases in which the recovery medium isn't the final assay medium, the cells are harvested by gently centrifugating again and resuspended in the actual assay medium. In a last step, the actual cell number is determined in order to be able to correct for the final cell number to be seeded per well. Seeding is achieved either with the help of multichannel pipettes or appropriate bulk reagent dispensers. We did not observe mechanical disruption of the cellular integrity even when using quite fast and rigorous seeding velocities so far. This robustness most likely reflects the lower susceptibility of the restricted number of cell types used in our lab and the typically, quite harsh conditions employed to generate monodisperse single-cell suspensions during the subculturing. As other cell systems may behave differently, multichannel pipettes and standard, bulk reagent dispensers can be adjusted for lowered addition speeds if necessary. Still, one may have to compromise for irregularities in plating quite low, exact cell numbers not because of a reduced precision of the dispensing tools but because of the fact that some cells rapidly start to settle in the seeding bottle already – in which case a more rapid dispensing helps greatly. Finally, the cells are seeded into the appropriate microplates, which are available in a wide variety of formats, materials, colours and well properties to suit the needs of the actual assay. We also noted little, if any, unresolvable problems even when prolonged incubation times are needed – given an uncompromised functionality of the incubator. However, this certainly has to be checked on a case-by-case basis to avoid any evaporation-related edge effects or a more general interference of the propagation procedure with proper cell function.

6 Assay Culture Conditions

When adapting cell-based assays to a high-density plate format, careful assay development becomes very important. Time and resource considerations are adding even more to this importance as increasing the numbers of wells to be tested asks for shortenings, modifications and even partial automation of certain aspects of the existing lab routines. However, as challenging as it may appear to put a lab's

existing low-throughput assay procedures on the test bench, it's scientifically highly rewarding in general. Chances are very good to come up with improved, cost-effective methods ensuring robust, reproducible and accurate assay performance and statistically proven and significant results. To implement a miniaturised cell-based assay, it is always a good starting point to immediately consider a final assay volume of 50 or 20 μL in the standard 96- or 384-well format – proportionally scaling down the amounts and volumes of all reagent additions. And it is also a good idea to ask whether several reagents can be combined and given in a lower volume via a single-addition step. It has to be shown that these variations do not affect the quality of the assay results – yet again, this is something easily tested in the microplate format. The same holds true for variations in the sequence in which the reagents are added. Finally, it is worthwhile to also check the feasibility of working at room temperature using HEPES as supplement to address pH issues if needed. With increasing familiarity to these procedures, one may notice that going for a 5 μL volume in a small-volume 384-well microplate works just as well and that one also reached the volume conditions needed for the 1536-plate format.

7 Cell-Based Assay Development

Aside from the often neglected basic cell culture routines and mandatory tool quality assessments, three major topics have to be addressed systematically in order to improve the quality of a miniaturised cell-based assay, i.e.

- Evaluation of the actual assay performance
- Demonstration of the reliability of the assay results with the help of appropriate statistical methods
- Choice of the detection method

The ***evaluation of the actual assay performance*** aims at the determination of the sensitivity, specificity and accuracy limits of a given assay in order to solve potential issues resulting from the expression levels, the exclusiveness and the exact quantity with which a given target or phenotype can be detected and measured in a particular cell model. Once a sufficient sensitivity is given, the general approach requires the assessment of key assay parameters and combinations thereof to detect potential interdependencies and rule out possible interferences. In doing so, the inclusion of proper high and low controls is of utmost importance. In addition, one is well advised to also run the original assay protocol in parallel in order to identify potential basic tool quality issues while also demonstrating the validity and comparability of the results. Yet the key to success results from identifying the range over which the assay read-out is proportional to the amount target present or phenotype observed. Once more, this is something that can easily be done by plating different amounts of cells into the same microplate and testing the influence of a particular parameter or condition of interest. In addition multiple replicates can be run smoothly, and – almost effortlessly – all tests can be repeated

independently for several times to demonstrate reproducibility (see Schulze et al. (2015) for an example). This strategy is also of great help when finally defining a standardised assay routine.

No matter what read-out technology is used, each assay has to deliver highly consistent data in the end. Thus, the ***demonstration of assay robustness and reproducibility*** is an obligatory part of all cell-based assay development efforts. From a practical point of view, this primarily asks for setting up cell-based assays with minimal variations in both intra- and inter-assay results. This is achieved best by using microplate-based systems to demonstrate that multiple replicate values do not substantially differ both on the very same test plate and additional plates prepared in parallel as well as during independent follow-up experiments performed the next day(s). In any case, the routine calculation of the standard deviation (SD; σ) of mean minimal and maximal signals becomes mandatory. These values are used to demonstrate that the discrimination power of a given assay is sufficiently high enough to unambiguously prove that a datapoint within a particular assay window significantly differs from the inherent assay noise. Once more, the usual calculation of signal-to-background S/B ratios should be used as a very first reference point only as it does not take the all-important assay variability into account at all. This can only be achieved when calculating the Z' value (Zhang et al. 1999), a statistical measure incorporating both the variability in the high and low control values and the S/B of an assay to better assess the actual size of the assay window (see Fig. 1b). The Z' value is a dimensionless factor and calculated the following way:

$$Z' = 1 - \frac{3\text{SD High control} + 3\text{SD Low control}}{(\text{Mean High control} - \text{Mean Low control})}$$

Although first published and used for screening purposes, the authors rightly point out that it can be used to describe the quality of any given assay as Z' values are determined from high and low controls only. As with other statistical parameters, however, larger data sets, i.e. large numbers of high and low control values, result in improved accuracy. The larger the Z' value achieved, the higher the data quality. In the end Z' values >0.5 are indicative of excellent assay performance.

With regard to the ***choice of the appropriate detection method***, quite a number of highly sensitive and accurate so-called homogenous read-out formats are available today. The term homogenous describes add, mix and measure technologies that allow for quite a significant reduction in the number of handling steps, thus significantly reducing the overall workflow of a microplate-based cellular assay. Given the fact that the ability to perform liquid handling and washing steps significantly decreases with assay miniaturisation, this is of particular importance. Furthermore, a thorough understanding of their measurement principles, specific needs and peculiarities is necessary to avoid measurement-related artefacts (i.e. potential false-positive and false-negative signals). To this end, the use of a second, orthogonal read-out format using an unrelated measurement principle has

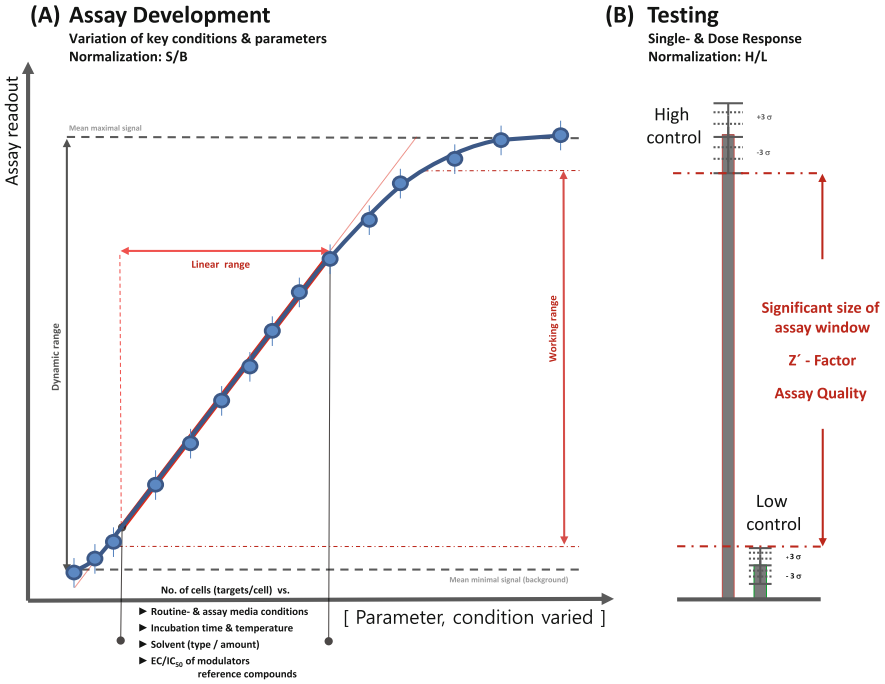

Fig. 1 **(a) Variation of key parameters and conditions** in order to establish the linear range of a cell-based assay. Some definitions: the linear range comprises the optimal region in which the actual assay read-out is proportional to a particular cell number (i.e. targets/cell) and the parameters/conditions varied, respectively. In contrast to the linear range, the dynamic range of an assay is defined as the area between the mean maximal and mean minimal signals (*background*). The crucial working range is obtained by subtracting 3 standard deviations (SDs; σ) from the maximal signal and adding 3 SDs to the minimal signal/background. Any value within the working range can be considered statistically significantly different with 99.7% confidence. (**b**) In cases in which **high and low controls** are **available only**, the calculation of the actual size of the assay window follows exactly the same procedure used to determine the working range of a given assay. In both cases it is obvious immediately that the degree of variation in the highest and lowest signals determines the actual assay quality: even assays with quite low S/B values can be evaluated with high precision once their maximal and minimal values do not oscillate very much – something generally achieved in a straightforward manner when switching from manual well-to-well pipetting to highly accurate electronic multichannel pipettes and dispensers

become a highly rewarding and mandatory routine to corroborate the results obtained.

Reporter gene assays rank first when it comes to studying and monitoring the seemingly innumerable number of cellular processes and signalling pathways in a cost-effective manner, although at the expense of having to deal with a time-delayed read-out due to the transcriptional and translational processes involved. The cell type of interest is either transiently or stably transfected to allow for the expression of an easily and sensitively detected reporter protein in response to a certain stimulus or process.

Still, the use of ***firefly luciferase*** (De Wet et al. 1985) (*luc*) predominates the field, and the number of applications described outperforms those of many other reporter proteins with enzymatic activities known.[11,12,13] Luciferase has been cloned in 1986 already and generates light with an emission peak at 562 nm by catalysing the conversion of luciferin to oxyluciferin in an ATP-dependent manner. Today, improved assay reagents are available, which prolong the duration of the luminescent signals from initially 1 to 5 min to hours so that quite a number of microplates can be processed in parallel without loss in signal intensity. In addition, the availability of different luciferase reporters (i.e. firefly and *Renilla* luciferase (Parsons et al. 2000)) can be used to set up dual luciferase reporter systems in which the second reporter notifies of unspecific, unrelated cellular processes, which likewise interfere with reporter protein expression, production and activity. The *Renilla* enzyme uses another substrate, i.e. coelenterazine instead of luciferin, with differing kinetics and spectral properties.

A similar well internal referencing capability comes with the combination of ***ß-lactamase*** (Moore et al. 1997) (*BLA*) as a reporter protein and a cell-permeable, artificial substrate that is composed of two dyes with different spectral properties linked together via a beta-lactam ring. Upon cell loading, this substrate becomes trapped intracellularly at physiological pH. Excitation at 409 nm allows for fluorescence resonance energy transfer (FRET) between a donor (coumarin) and an acceptor dye (fluorescein) leading to a green emission signal at 518 nm. Once expressed, BLA enzyme activity separates both FRET partners and abolishes signal generation as the resonance energy transfer is highly sensitive to smallest changes in the distance between the donor and the acceptor partners. Nevertheless, a blue emission signal at 447 nm can be observed upon excitation at 409 nm instead. Thus, the calculation of the ratio between the 447 nm and the 518 nm emission signals can be employed to eradicate well-to-well cell variations and to detect fluorescence interference events. Although this procedure is leaving the cells intact for subsequent studies, it should be noted that the BLA reporter currently used is a genetically engineered version devoid of any signal peptide and resides inside the cell, whereas the prototype enzyme, encoding the bacterial ampicillin resistance marker, belongs to the class of secreted reporter proteins which can be utilised to quantify reporter gene activity in supernatants. In our lab, both luc ad BLA reporter systems are used in an orthogonal manner to either confirm screening hits or exclude artefacts resulting from transcriptional or translational processes as well as fluorescence interference.

Any over-amplification of weak primary signals by luciferase (Hill et al. 2001) or BLA enzyme activity has to be considered seriously, as the magnification of weak, basal responses may either mask partial activity effects or lead to an

[11] For chloramphenicol acetyltransferase (CAT), see Devinoy et al. (1991).

[12] For secreted alkaline phosphatase (SEAP), see: Cullen and Malim (1992).

[13] For secreted urokinase, see Langer et al. (1995).

overestimation of the relevance of a signal in certain cases – something that holds true for any read-out based on reporter genes with enzymatic activity, however.

The use of the inherently fluorescent ***green-fluorescent protein*** (Remington 2011) (*GFP*) and its broad range of spectral variants represents the most recent, highly attractive and cost-effective reporter protein approach to monitor the plethora of cellular processes both in a spatial and temporal manner. The natural, unmodified GFP protein emits a green signal at 509 nm following excitation at 395 nm. At first the step excitation triggers an autocatalytical cyclisation of three adjacent amino acids. Final chromophore maturation is achieved in the presence of molecular oxygen which generates a highly conjugated π-electron system, deeply buried in a rugged and compact ß-barrel protein structure. The small cylindrical shape and the bioluminescent properties of GFP make it an ideal tool for tagging and monitoring cellular proteins without the need for any other additional detection reagents. Furthermore, the availability of GFP versions with different spectral properties lead to a rapid explosion in the number of applications. In addition, many staining procedures based on the use of fixed cells with fluorescently labelled antibodies introduced in the 1970s have been replaced this way. Although a GFP tag adds another 27 kd to the protein of interest, it rarely interferes with the functionality or localisation of the fusion partner. On the other hand, the fusion partner and its subcellular location can affect the folding and stability of GFPs even if the codon usage is corrected to reflect the actual system of choice. This certainly asks for an independent and unambiguous confirmation of the results obtained with a GFP experiment by using an appropriate antibody (Snapp 2008) whenever possible. Quite some caution is necessary as well when employing different GFP versions or GFP/luciferase combinations in FRET or BRET experiments, which aim at studying the intra- and intermolecular interactions between two or even more partners. Key experiments to be performed comprise of quite a number of variations of both donor and acceptor molecule ratios with the help of transient transfection experiments to avoid measuring unspecific bystander effects only,[14,15] even though the introduction of protein-destabilising domains into GFPs leads to a reduction of high basal GFP signals due to the extraordinary half-life of the native protein (>36 h).

Yet, in the majority of cases, the generation and propagation time of most of the signals involved in cellular processes and signalling pathways is much more rapid[16] to be followed up by transcriptional responses in a meaningful manner. Both the shortened time between stimulus and read-out and the actual scale of the measurement window require specialised detection and read-out formats – see Wunder et al. (2008) for a comprehensive discussion of measurements involving Ca++ signalling and ion channel activity.

[14] For a discussion of FRET artefacts and signal proper, see (Vogel et al. 2006).

[15] For a thorough examination of potential BRET-related artefacts, see Marullo and Bouvier (2007).

[16] That is, from milliseconds to seconds and from a few minutes to a few hours

Enzyme fragment complementation (EFC) can also be used to interrogate rapid and distinct interactions between two protein partners in a direct and speedy manner (Eglen 2002). In principle, a small enzyme donor peptide (ED) is fused to the first and an enzyme acceptor protein (EA; optimised for ED affinity) to the second interacting partner. Any stimulus- or modification-dependent contact between the two interacting proteins brings EA and ED into close vicinity, thereby generating a fully functional ß-galactosidase enzyme.[17] The enzyme activity is quantified with the help of an appropriate substrate, and enzyme cleavage results in the generation of a chemiluminescent signal – the signal intensity achieved of being compatible with all microplate formats. In addition to the study of protein-related interactions including protein (*trans*-) location and degradation studies,[18] the very same approach can also be used in competitive immunoassays. Here the analyte of interest is conjugated to the small enzyme donor (ED) peptide and recognised by an antibody fused to the enzyme acceptor (EA). This allows for a very rapid and highly sensitive detection cytosolic 2nd messengers as well as protease activities[19] in cell-based assays. Again, a careful determination and titration of the expression levels of the interaction partners or the amounts of analyte used are mandatory to prevent artefacts resulting from supra-physiological expression levels and to determine the linear range of the assay in order to obtain meaningful results.

An equivalent, though non-enzymatic, way of analysing molecular interactions in cells in vitro can be achieved with the help of ***time-resolved fluorescence resonance energy transfer*** **(TR-FRET)** technologies based on the use of rare-earth (Eu^{3+}/Tb^{3+}) chelates[20] or cryptates.[21] In its early days this approach has been used to primarily set up competitive immunoassays as well. In this case, an antibody to a protein or analyte of choice is labelled with a lanthanide molecule as FRET donor to allow for fluorescence resonance energy transfer (FRET) to an acceptor fluorophore either directly or indirectly linked to the protein or analyte recognised. Because lanthanide labels are not fluorescent on their own but do emit a strong, long-lasting fluorescence signal following excitation, the actual assay background noise is dramatically reduced especially when combined with time-resolved detection. Thus, this approach has widely replaced most albeit not all other fluorescent molecules used in FRET studies. Today, a wide variety of applications are known, and an ever-increasing number of assays are being developed for use in

[17] A process called complementation that has been reported in 1965 already, see Ullmann et al. (1965).

[18] See http://www.discoverx.com/technologies-platforms/enzyme-fragment-complementation-technology/pathhunter-efc-cell-based-assay-platform.

[19] For more details, see http://www.discoverx.com/technologies-platforms/enzyme-fragment-complementation-technology/hithunter-efc-biochemical-assay-platform.

[20] For dissociation-enhanced lanthanide fluorescent immunoassays (Delfia) marketed by PerkinElmer, see http://www.perkinelmer.com/catalog/category/id/delfia%20trf%20assays%20and%20reagents.

[21] The use of lanthanide cryptates is called HTRF (**h**omogeneous, **t**ime-**r**esolved **F**RET) and marketed by the French company Cisbio http://www.cisbio.com/other.

homogenous, highly sensitive, state-of-the-art detection methods in the field of cell signalling.[22] Moreover, companies like Cisbio also provide different toolbox reagents in order to evaluate, set up and implement assays meeting actual research needs,[23] and it is up to the scientific imagination of individual researcher to envisage, develop and make use of completely novel assay types. In 2010, for example, the use of small protein tags for covalent dye labelling of proteins in living cells has been reported in a *HTRF* assay to detect, measure and quantify the binding of fluorescently labelled ligands in living cells (Zwier et al. 2010). In the meantime, this approach has been extended to high-throughput studies aiming at the determination of ligand-binding kinetics in real time and with high quality (Schiele et al. 2014). In addition, a multicolour, multiplexing HTRF approach has been published (Cottet et al. 2015) just recently, which aimed at the recording and quantifying of both the ligand-binding properties and the interaction of three different receptors in living cells simultaneously.

8 High Content Analysis: Getting the complete picture

Microscope-based assay techniques allow for the simultaneous observation, visualisation and quantitation of cellular processes in a temporal and spatial manner. In comparison to cell-based assay formats delivering only a single read-out, this inherent multiplexing capability represents a major advantage and greatly contributed to the development of *high-content analysis* (Denner et al. 2008) (*HCA*) systems. The technology builds on the advances in the fields of digital photography and microscopy especially with regard to the rapid, large-scale acquisition of high-resolution cellular imaging data. Today, a number of highly integrated, automated confocal microscopy units are available including microplate handling systems to increase throughput for large-scale *high-content screening* (Hoffman and Garippa 2007) (*HCS*) approaches,[24,25,26] in addition to lower throughput, smaller versions[27,28,29] and multimodal plate readers with imaging capabilities.[30]

[22] For more details, see http://www.cisbio.com/other/drug-discovery as well as http://www.perkinelmer.com.

[23] See http://www.cisbio.com/drug-discovery/toolbox-products-and-services.

[24] http://www.perkinelmer.com/pages/020/cellularimaging/products/opera.xhtml

[25] http://www.gelifesciences.com/webapp/wcs/stores/servlet/catalog/en/GELifeSciences-us/applications/hca-imaging-systems-and-software

[26] http://www.lifetechnologies.com/order/catalog/product/ASN00003L

[27] http://www.perkinelmer.com/pages/020/cellularimaging/products/default.xhtml

[28] http://www.moleculardevices.com/systems/high-content-imaging/imagexpress-micro-xls-widefield-high-content-analysis-system

[29] http://ttplabtech.com/phenotypic-screening/acumen/

[30] http://www.biotek.com/products/imaging/cytation5_cell_imaging_multi_mode_reader.html

Whereas the reporting format of a standard microplate reader consists of a single average value per well,[31] an HCA reader is capable of reporting multiple values not only in the well average format but also for every single cell depending on the particular resolution used.[32] Thus, the HCA format opens entirely novel and fascinating dimensions to study the complexity of cellular processes as virtually any phenomenon pictured with the help of a digital camera can be gathered and subjected to quantitative analyses and qualitative examinations (Luense et al. 2015). To this end, the use of highly sophisticated image analysis software tools allows for the recognition, classification and quantitation of distinct cellular patterns and a precise contextual interpretation of the datasets acquired. The software groups individual cellular processes according to quantifiable image properties such as size, shape, pixel intensity and positional information for each of the wavelengths used. Next, specific threshold settings are defined and implemented to reduce background noise – in addition to the ones possibly obtained with the help of confocal techniques. One has to keep in mind, however, that in contrast to the well-defined, physical signal characteristics of a "conventional" measurement method, the HCA signal is derived from software algorithms, which have to be newly developed each time and specifically modified to fit and accurately report the cellular process studied. Thus, great care has to be taken in the development and definition of these algorithms, and one is well advised to validate the results obtained in comparison to conventional gold standard assays and read-outs whenever they are available. Admittedly though, highly user-friendly and excellent image analysis tools are available from multiple (instrument) vendors to meet this challenge, and as it is out of the scope of this paper to provide a detailed insight into their functionalities, the reader is referred to Zwier et al. (2010) for more detailed information. In addition, all this is greatly facilitated by the massive increase in data storage and retrieval capabilities of modern storage systems and the processing power of current computers.

From a cell biologist's point of view, the quality of the cells used for HCA assays is key to obtaining meaningful data – again pointing out the importance of standardised routine and assay culture procedures discussed already. Furthermore, the use of frozen cells greatly facilitates the setting up of robust and reliable HCA assays, especially considering the additional amount of time that comes along with regard to the numerous handling steps involved in the labelling[33] (Larsson 1988a) and/or fixation (Larsson 1988b) of cells prior to any microscope-based analysis. In this respect the use of autofluorescent proteins for live cell imaging greatly expands the existing assay repertoire.

[31] That is, average signal intensity, which can be the product of multiple sub-parameters of course

[32] In a sense permitting population studies otherwise known from fluorescence-activated cell sorter (FACS) analysis

[33] Molecular Probes® Handbook – A Guide to Fluorescent Probes and Labeling Technologies; the printed version is available free of charge via http://www.lifetechnologies.com/de/de/home/references/molecular-probes-the-html.

Clearly, state-of-the-art cell-based functional assays offer a wealth of additional information compared to biochemical, target-based characterisation approaches. Nonetheless it has always been clear and is becoming more important over the last two decades again that 2D cell culture models provide very little information regarding the actual behaviour of cells in a 3D tissue environment consisting of many different cell types and various mechanical/physiological and morphological barriers (Breslin and O'Driscoll 2013). This led to a renaissance in the development and *use of 3D cell culture models* beyond traditional, biotechnological tissue engineering efforts – last but not the least due to the enormous progress in automated microscopy just alluded to. Today quite a number of different 3D cell culture in vitro models are available to scrutinise basic scientific hypotheses related to the regulation of tissue morphogenesis (Yamada and Cukierman 2007) in addition to also being used as cancer models in translational research (Thoma et al. 2014) and drug discovery efforts (Friedrich et al. 2009; Hirschhaeuser et al. 2010). And although there is quite a considerable debate with regard to the robustness and reliability of signal generation as well as the actual scope of applications for different models used (Fennema et al. 2013; Matsusaki et al. 2014), quite a number of publications document the pronounced usefulness of this approach as exemplified by Burgstaller (et al. 2013) and Wenzel et al. (2014). Of particular note is one approach that validated and used multilayered cultures of primary human fibroblasts, mesothelial cells and ECM molecules as a model system for human ovarian cancer microenvironment (Kenny et al. 2015).

As with every assay technique, whether one opts for the use of a "traditional" 2D monolayer culture or the application of advanced 3D culturing method, the determination of the appropriateness of the model comes first – keeping either the scientific hypothesis to actually test in mind or its planned probe identification/drug development purpose. Yet one does not only have to be aware of the limits of the particular model itself but also of its technical complexity – no matter how attractive a specific approach may seem in the first place. Once these questions have been answered, with scientific ambition the cell-based assay development strategies outlined here come into play and can be used to ensure the provision of significant, reliable and high-quality data.

However, according to an epidemiologist's challenging paper (Ioannidis 2005):

- The smaller the number of studies conducted and their effect size
- The greater the number of and the less the selection of relationships tested
- The greater the flexibility in the design

the less likely the results will be true. As the use of cells in microplate assay formats greatly facilitates dealing with these issues in an appropriate manner, the microplate approach has to be regarded as state-of-the-art technique for scientific research. The use of appropriate assay development and implementation strategies greatly facilitates statistically sound and rigorous testing of assays by providing the

researcher with the opportunity to address the multitude of factors affecting the actual assay results in a much more systematic fashion and in a timely manner. Furthermore, it will allow for setting up more robust and reliable test systems that ensure the confidence in the statistical significance of the actual data generated especially in the field of in vitro cell-based assays.

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Translational In Vivo Models for Women's Health: The Nonhuman Primate Endometrium—A Predictive Model for Assessing Steroid Receptor Modulators

Ov Daniel Slayden

Contents

Abstract

Macaques and baboons display physiological responses to steroid hormones that are similar to those of women. Herein, we describe various uses of nonhuman primates for preclinical studies on menstruation, endometriosis, and as a model system to evaluate reproductive therapies and contraceptives. Our goal is to outline the strengths of the nonhuman primate model for studies leading to improved therapies for women.

Keywords

Animal models · Endometriosis · Menstruation · Nonhuman primate · Progesterone receptor modulators

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1 Introduction

Preclinical studies on novel therapies frequently require testing in suitable models, and rodent models are often the first choice for study because of cost. However, reproductive physiology in rodents is strikingly dissimilar from women, and therapeutic interventions in rodents often fail to fully replicate in clinical trials (Pullen et al. 2011). In contrast, macaques share approximately 95% of genetic coding sequence identity with humans (Magness et al. 2005). Moreover, most responses to hormonal therapies in women are fully reproduced in nonhuman primates (NHPs) (Shively and Clarkson 2009). Rhesus and cynomolgus monkeys (*Macaca mulatta* and *M. fascicularis*) have been particularly useful in biomedical studies because of their moderate size. Although strikingly larger, baboons present spontaneous adenomyosis (Barrier et al. 2004), endometriosis (Cornillie et al. 1992), and endometrial hyperplasia (Barrier et al. 2007) similar to women. Moreover, both macaques and baboons can display polycystic ovarian syndrome-like features and are suitable models for interventional therapies relative to ovarian-based infertility (Fraser and Duncan 2009).

Macaques and baboons can be trained to accept a wide variety of manipulations that facilitate drug administration and sample collection. For example, steroid receptor modulators including progestins and progesterone receptor antagonists (PRA) have been administered to macaques transvaginally (Hodgen 1985), orally (Tarantal et al. 1996), systemically by injection (Slayden et al. 1998), and by subcutaneous implant (Brenner et al. 2006). Because the action of these agents on endometrial histology and physiology has been well characterized, the assessment of endometrial histology after administration of novel agents has become a priority in the screening of new therapies before development of clinical trials.

Old World monkeys display menstrual cycles and reproductive tract anatomy similar to women (Brenner and Slayden 1994). Hysterectomized NHPs, like women, continue to undergo normal ovarian cycles indicating that uterine feedback is not required for ovarian function (Metcalf and Livesey 1988; Molskness et al. 2007). The only ovarian factors absolutely required to elicit cyclic responses in the primate endometrium are the ovarian steroids estradiol (E_2) and progesterone (P_4) (Hisaw and Hisaw 1961). The uterine cavity of NHPs is lined by a thick glandular endometrium with four distinct endometrial zones, surrounded by a thick muscular wall or myometrium (Bartelmez 1933, 1951). The upper three of the endometrial zones are frequently referred to as the endometrial functionalis; the zone adjacent to the myometrium is referred to as the basalis zone. The functionalis zone in NHPs is vascularized by unique "spiral" arteries, whereas the basalis zone is vascularized by the basilar arteries. The functionalis zones are typically shed during menstruation and are regenerated each cycle from the basalis zone. The histology and hormone regulation of these zones have been well characterized and provide the basis for use of these animals for studies assessing novel therapies on reproductive tract physiology (Slayden and Keator 2007).

Fallopian tube histology is also similar between NHPs and women. Compared to ovariectomized individuals, estrogen stimulates differentiation of the tubal

epithelium into a ciliated and secretory state (Brenner and Slayden 1995). Progesterone gradually suppresses estrogen-stimulated oviductal differentiation. In contrast to the oviduct and endometrium, the cervix and vagina of different nonhuman primate species vary extensively (Hafez and Jaszczak 1972). For instance, the cervix of women is typically short with a straight canal; baboons display a longer cervix, also with a straight canal. In contrast, macaques possess a large cervix with a striking sigmoidal flexure. The torturous nature of the cervix of macaque complicates transcervical procedures including noninvasive biopsies and hysteroscopy. Also like the oviduct, the cervix of macaques displays ciliated cells that increase in number during the proliferative phase of the menstrual cycle (Jaszczak and Hafez 1973).

2 Use of Artificial Cycles

The menstrual cycle consists of an estrogen-dependent "follicular" or "proliferative phase" and a P_4-dependent "luteal" or "secretory phase." NHPs including macaques (Slayden and Brenner 2004), vervets (Carroll et al. 2007), and baboons (Cox et al. 2000) have been ovariectomized and treated with subcutaneous capsules that release E_2 and P_4 to recreate the pattern and level of serum E_2 and P_4 that occurs during the natural cycle (Slayden and Brenner 2004). A standardized artificial cycle protocol (Slayden and Brenner 2004) has been developed for macaques where ovariectomized animals are treated with a subcutaneous 3–5-cm E_2-filled Silastic capsule for 14 days and then leaving the E_2 capsule in place, a 6-cm subcutaneous P_4-filled capsule is placed for 14 days. Removal of the P_4 capsule results in menstruation and completes the cycle.

The "artificially cycled" animals display endometrial histology that is identical to the natural cycle (Slayden and Brenner 2004). For instance, E_2 during the artificial follicular phase drives epithelial cell proliferation and P_4 during the artificial luteal phase-stimulated glandular secretory differentiation. It is noteworthy that a brief period of glandular cell proliferation occurs in the endometrial basalis zone during the artificial luteal phase (Padykula et al. 1989).

Artificial menstrual cycles appear to be physiologically competent because it is reported that pregnancy can occur in ovariectomized artificially cycled macaques (Hodgen 1983). The artificial cycle protocol produces well-defined levels of E_2 and P_4, and therefore, these animals are useful for pharmacodynamic evaluation of steroid antagonists.

3 Induction of Menstruation

Old World NHPs including baboons and macaques menstruate at the end of each cycle similar to women. During menstruation the upper (luminal) half to two thirds of endometrium is shed. In the macaque, treatment of E_2-primed animals with P_4 (>1 ng/ml in serum), for at least 3 days, is required to prime the endometrium for

menstruation. Thereafter, withdrawal of P_4 stimulates endometrial expression of matrix metalloproteinase (MMP) enzymes in the upper endometrial zones (Rudolph-Owen et al. 1998; Slayden and Brenner 2006). The MMPs are capable of degrading the extracellular matrix and are the primary effectors resulting in dissociation of functionalis zone tissues.

The vervet also menstruates at the end of each cycle, but menstruation in this species is very light requiring vaginal swabbing for detection (Carroll et al. 2007). Much of our understanding of the anatomical events of menstruation was described by Markee (Markee 1940, 1948, 1950) who visualized menstruation in macaque with endometrial autografts placed in the anterior chamber of the eye. In several studies we transplanted the endometrium to subcutaneous and intra-abdominal ectopic sites of macaques. Sequential treatment with artificial cycle capsules releasing E_2 and P_4 in animals bearing these "autografts" results in histological changes and menstruation within the grafts similar to the endometrium in situ (Brenner et al. 1996).

We have extended the use of artificial cycles to specifically study menstruation (Rudolph-Owen et al. 1998; Slayden and Brenner 2006). Withdrawal of P_4 at the end of the cycle (with or without withdrawal of E_2) results in expression of endometrial matrix metalloproteinases that act to breakdown the endometrium during menstruation.

The timing of menstruation and the abundance of menstrual spotting and breakthrough bleeding can be assessed in monkeys trained to present for sampling by vaginal swab (Slayden et al. 2007). Monkeys can also be trained to accept vaginal tampons for the collection of menstrual flow (Shaw, Jr. et al. 1972), and the tampons analyzed for menstrual blood. This technique has been used to assess therapies on menstrual blood loss (Brenner and Slayden 2012).

4 Steroid Receptor Antagonists

Steroid hormones induce their effects on tissues via interactions with steroid-specific receptors including estrogen receptors (ESR), progesterone receptors (PGR), and androgen receptors (AR). Studies on hormonal regulation of steroid receptor levels have been accomplished in artificially cycled NHPs using classical binding studies (West et al. 1987), immunohistochemistry (Slayden and Brenner 2004), and molecular methodologies (Keator et al. 2012). Immunohistochemical studies strongly support older work that quantified ESR and PGR by binding assays. Localization of these receptors is tightly correlated with morphological changes during the cycle (Slayden and Brenner 2004). Expression of these receptors, therefore, both indicates responsiveness to hormone stimulation and provides a sensitive marker for hormone action. Treatment of the E_2-primed endometrium with P_4, reduces ESR-1, PGR, AR, and membrane-associated progesterone receptors (Keator et al. 2012).

Many studies have treated NHPs with various steroid receptor antagonists including both estrogen receptor agonists (ERA) (Ethun et al. 2012) and progesterone receptor antagonists (PRA) (Chwalisz et al. 2006). Treatments have included

daily injections (Slayden et al. 1998; Zelinski-Wooten et al. 1998b) as well as intrauterine administration (Brenner et al. 2010). When administered in the artificial luteal phase of the cycle, potent PRAs including mifepristone (Slayden et al. 2007), ZK 137 316 (Slayden et al. 1998) and ZK230-211 stimulate menstruation of similar duration observed after P withdrawal. Treatment with PRAs disrupts the normal endometrial morphology, which at high doses results in endometrial thinning which has been termed a noncompetitive antiestrogenic effect (Neulen et al. 1996; Wolf et al. 1989). As expected, treatment with potent antiestrogenic compounds disrupts E_2-induced increases in these receptors and at higher doses results in a state similar to no hormonal treatment (Carroll et al. 2008). Treatment of artificially cycled macaques with potent PRAs, like mifepristone, results in apparent overexpression of ESR-1, PGRs, and AR (Slayden et al. 2001b). Mifepristone in particular increases AR expression to detectable levels in glandular epithelium.

In several studies we have used the induction of menstruation as a bioassay for evaluating PRA potency. Conversely, treatment, with potent progestomedins like levonorgestrel blocks normal cyclic menstruation, can often induce unwanted irregular menstruation-like bleeding and spotting often referred to as "breakthrough bleeding" in both macaques and women (Hickey et al. 2006).

The effects of various regimens of mifepristone and some other PRA compounds including ZK 137 316 and ZK 230 211 on the endometrium of rhesus macaques have been reviewed (Brenner et al. 2002). Acute administration of these compounds during the secretory phase blocks the action of P_4 and induces menses similar to P_4 withdrawal. When an effective P_4-blocking dose of a potent PRA like ZK 230 211 is administered daily throughout the cycle, secretory phase progestational differentiation of the endometrium is completely suppressed, but in addition, some of the effects of E_2 are inhibited so that in many of the animals, the glandular epithelium becomes atrophied, the stroma becomes dense, mitotic activity is suppressed, and endometrial thickness is greatly reduced. These endometrial antiproliferative effects also occur when ovariectomized animals are treated only with E_2 plus PRA indicating that the effect is selectively "antiestrogenic," even though PRAs do not bind to the ER (Wolf et al. 1989). In addition, PRA treatment leads to stromal cell atrophy, stromal compaction, and hyalinizing morphology within the spiral arteries, all in the presence of physiologically adequate serum E_2 levels. Similar effects were found after long-term treatment of intact cycling macaques with low doses of RU 486 (Grow et al. 1996). Also, in the endometrium of women treated chronically with low doses of RU 486, glandular mitosis was inhibited and stromal compaction was induced (Baird et al. 2003).

Administration of PRAs prevents pregnancy (Batista et al. 1992; Borman et al. 2003; Katkam et al. 1995; Nayak et al. 2007; Slayden and Keator 2007; van Uem et al. 1989; Zelinski-Wooten et al. 1998a). Mifepristone, ZK 137 316, onapristone, and ulipristal have been shown to block fertility. Currently, ulipristal acetate and mifepristone are available for emergency contraception (Carbonell et al. 2015; Fine et al. 2010; Gemzell-Danielsson et al. 2013; Glasier et al. 2010; Koyama et al. 2013; Langston 2010; Mozzanega et al. 2014). Under these conditions, the lowest possible dose is desired, and chronic treatment of women

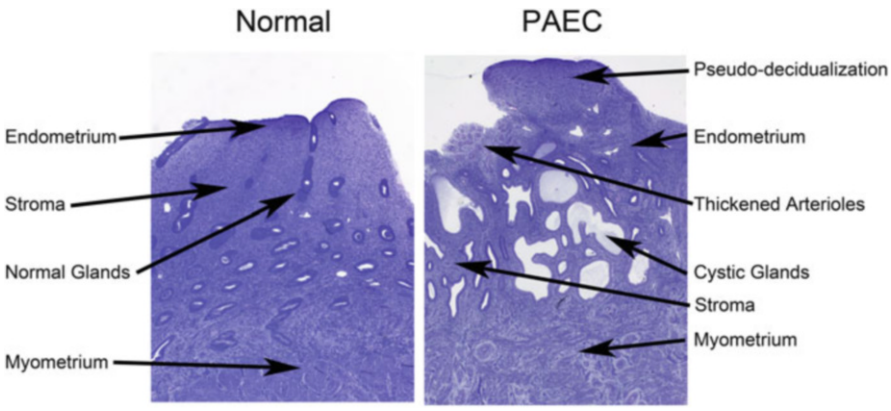

Fig. 1 Photomicrograph of H&E stained endometrium of rhesus macaques. Normal endometrium shows typical mid-cycle morphology with tubular glands. PAEC was caused by treatment with low-dose PRA. Note the cystic glands, hypertrophied spiral arteries, and pseudodecidualized stroma in the upper 1/3 of the endometrium

with low-dose PRAs often results in endometrial thickening. Resultant histology presents as cystic dilatation of endometrial glands, pseudo-decidualization, and thickening of the spiral arterioles. Collectively these changes have been termed "PGR modulator-associated endometrial change" (PAEC) (Williams et al. 2007). Our early studies in NHPs failed to detect PAECs and reported that PRA treatments resulted in endometrial thinning (Brenner et al. 2002; Chwalisz et al. 2000b). This outcome appears to be the result of the high (menstruation inducing) doses of PRA administered during these trials (Slayden et al. 1993; Slayden and Brenner 1994; Wolf et al. 1989). Recent experiments with low-dose PRAs in cycling NHPs demonstrate glandular cysts with significant secretion and spiral artery morphology similar to PAEC in women. Figure 1 shows a low-power image of rhesus macaque endometrium showing PAEC after treatment with low-dose PRA. While there is no evidence that PAEC in women leads to atypical hyperplasia or endometrial carcinoma, the striking change in endometrial appearance can be alarming and has delayed development of effective progesterone receptor modulator-based contraceptives.

Treatment with PRAs induced striking increases in endometrial ERα, PR, and AR. AR in particular was elevated in both the endometrial stroma and in the glandular epithelium of the functionalis zone. The mechanism through which PRA suppresses E_2 effects on cell proliferation has not been fully explained (Slayden et al. 1993, 1998, 2001a; Slayden and Brenner 1994). Hodgen's laboratory reported that RU 486 treatment resulted in excessively elevated ESR, and they suggested that elevated ESR could produce a super-estrogenized state (Neulen et al. 1990, 1996). Since super-physiological doses of E_2 are reported to be inhibitory to endometrial growth (Neulen et al. 1987), then overexpression of ESR could result in a similar antiproliferative action. However, as mentioned above, PRA

treatment also elevates endometrial AR. Because androgens are known to inhibit some estrogen-dependent endometrial growth, elevations in AR induced by PRA could allow endogenous androgens to suppress proliferation.

PRAs can induce striking changes of the spiral arteries (Slayden et al. 1998, 2001a), which initially appears hypertrophied and then degrade with chronic administration. Endometrial abnormalities are also reported for women after PRA therapy. These changes in women do not fully replicate those seen in NHPs, but both are associated with non-mitotic glandular dilation and arterial abnormalities (Baird et al. 2003; Williams et al. 2007). These vessels, which are unique to the primate endometrium, are primary targets for PRA action even though the endothelium and smooth muscle of the arteries lack PR. Only the perivascular stromal cells express PR and AR, and these may be the cells most affected by PRAs. The damage to the spiral arteries, which includes narrowed lumens and hyalinized walls, probably leads to reduced vascular perfusion which could play an important role in the endometrial antiproliferative effect (Chwalisz et al. 2000a).

5 Induction of Endometriosis

Endometriosis is a gynecological disorder defined by the presence of endometrium-like tissues at "ectopic" sites outside the uterus (Burney and Giudice 2012). The predominant theory, the Sampson Hypothesis (Bricou et al. 2008; Sampson 1940), proposes that endometriosis arises from retrograde menstruation of endometrial fragments through the fallopian tubes. While this theory has not been unequivocally proven, there is substantial evidence that endometriosis-like lesions can be created in NHP models through endometrial transplantation. The limitations on clinical studies, and those using naturally occurring endometriosis in NHPs, have resulted in the development of strategies to create endometriosis-like lesions in non-primate animal models (Grummer 2006). Spontaneous endometriosis is rare in most primate colonies (Story and Kennedy 2004), and there are no reliable noninvasive screening technologies to identify animals with the disease (D'Hooghe et al. 2009). Induction of endometriosis in the baboon was initially described by D'Hooghe (D'Hooghe et al. 1994; D'Hooghe et al. 2009), and although the efficiency of the baboon model was recently challenged (Dehoux et al. 2011), it has been utilized extensively by Fazleabas and coworkers over the last decade at the University of Illinois at Chicago and Michigan State University (Fazleabas 2006; Fazleabas 2010; Harirchian et al. 2012).

6 Conclusions

Old World NHPs, especially macaques and baboons, continue to provide a unique and valuable animal model for experimentally testing the role of novel receptor ligands. The close physiological and anatomical responses of these species to

women provide an outstanding system for evaluating novel therapies for women's health.

Assessment of endometrial morphology combined with specific histological markers remains an excellent tool for rapidly assessing the effects of steroid hormone modulators on the endometrium. Treatment of NHPs with E_2 and P_4 results in hormonally driven switch points providing an objective assessment of receptor action. Morphological assessment will remain essential because distinct gradients in physiological response occur within the endometrium, and without accurate histological characterization, modern genomic approaches may fail to yield a definitive assessment of endometrial receptivity.

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Predictive In Vivo Models for Oncology

Diana Behrens, Jana Rolff, and Jens Hoffmann

Contents

Abstract

Experimental oncology research and preclinical drug development both substantially require specific, clinically relevant in vitro and in vivo tumor models. The increasing knowledge about the heterogeneity of cancer requested a substantial restructuring of the test systems for the different stages of development. To be able to cope with the complexity of the disease, larger panels of patient-derived tumor models have to be implemented and extensively characterized. Together with individual genetically engineered tumor models and supported by core functions for expression profiling and data analysis, an integrated discovery process has been generated for predictive and personalized drug development.

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Improved “humanized” mouse models should help to overcome current limitations given by xenogeneic barrier between humans and mice. Establishment of a functional human immune system and a corresponding human microenvironment in laboratory animals will strongly support further research.

Drug discovery, systems biology, and translational research are moving closer together to address all the new hallmarks of cancer, increase the success rate of drug development, and increase the predictive value of preclinical models.

Keywords

Mouse models · Patient-derived xenograft (PDX) · Preclinical oncology · Translational research

1 Introduction

Tumor biology research and preclinical drug discovery both depend heavily on specific in vivo disease models. Historically, basic research and drug characterization were based on a handful of preclinical tumor models from each indication. Given our current knowledge about tumor heterogeneity, we can now understand why results from studies with 2–3 lung cancer models could have been only lowly predictive for the clinical outcome and a risky development work was a burden to clinicians and patients.

Almost in parallel with the new millennium, processes have changed substantially. This has been driven by increasing costs for the clinical development in contrast to often disappointing improvements for the patients. Growing insight into the fundamental genetic basics of the disease through analysis of gene expression and mutations and the development of fascinating new technologies in genetic engineering and bioinformatics – key word systems biology – have provided the technical basis for this paradigm shift.

As consequence, primary pharmacology processes in preclinical cancer research have changed, and the elementary task is the establishment of the right model and access to appropriate tools for each step of the drug discovery process (as shown in Fig. 1). This also requires former single disciplines to work more and more together, forming a more and more integrated process of preclinical drug discovery.

2 Demands on Target Identification and Validation Models

Innovative technologies in target identification and validation have also changed the request on the disease models. Have been a small number of extensively characterized tumor cell cultures and mouse models the standard for many decades, the target-driven approaches now require models reflecting better the clinical situation. Genotype-dependent stratification of patient cohorts to predict efficacy

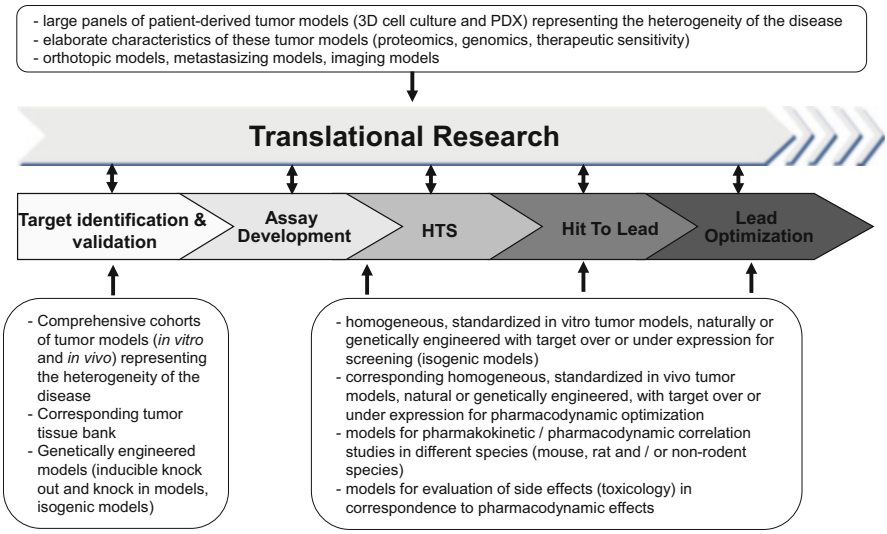

Fig. 1 Oncology drug development requires well-characterized panels of in vitro and in vivo tumor models, standardized analytical methods, as well as experimental settings to transfer bench-side targets into drugs for the clinic

of specific drugs is now seen as a prerequisite for the development of molecular-targeted therapies. Slamon and colleagues were the first using a cancer cell line panel to validate overexpression of Her2 as a predictive marker in breast cancer for the efficacy of Herceptin (Slamon and Pegram 2001). The lack of such studies in large cell culture panels in other indications has hindered the development of further epidermal growth factor receptor (EGFR) targeting drugs, i.e., gefitinib, cetuximab, or panitumumab. Initially, some unstratified studies failed, and only a posteriori genotype-dependent stratification of patient cohorts allowed predicting efficacy and further development of targeted therapeutics like cetuximab or panitumumab (Lièvre et al. 2006; Amado et al. 2008).

The requirements on new models for target identification and validation (TIV) include among others:

- Availability of large panels of tumor models (in vitro and in vivo) representing the heterogeneity of the disease
- Extensive data about the characteristics of these tumor models (gene and protein expression, gene amplifications, mutations, epigenetics, miRNA expression, histology, reference drug sensitivity)
- Corresponding databases containing all these information and tools allowing bioinformatic analyses
- Tumor tissue banks (frozen and paraffin-embedded tissue, tissue microarrays)
- Technology to generate genetically engineered models (inducible knockout and knock-in models, isogenic models)

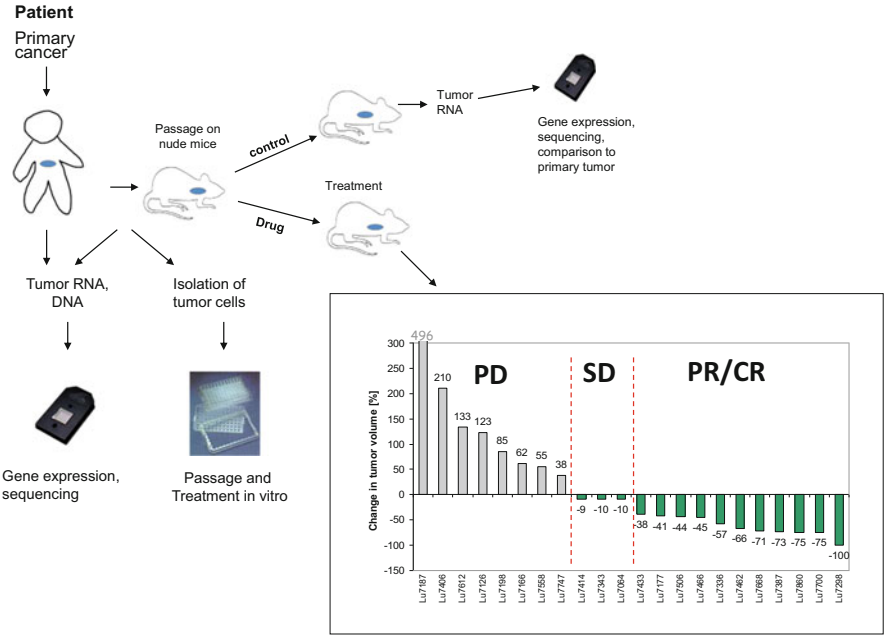

Fig. 2 Workflow of preclinical research using convenient PDX mouse models. Patient-derived material – expanded in mice – undergoes molecular biological and therapeutic screening. A waterfall plot distinguishes responder from nonresponder (Fichtner et al. 2008). RECIST criteria: *PD* progressive disease, *SD* stable disease, *PR*/*CR* partial or complete response

A key component of preclinical strategies is the so-called patient to mouse xenotransplantation model (PDX), established by transplantation of fresh patient material to immunodeficient mice (shown in Fig. 2). After a successful engraftment (growth to a tumor volume between 500 and 1,500 mm^3) within 2–6 months, the PDX can be used for serial transplantation over several generations (P2–P10). Expanded material can be used for drug response or biomarker studies, the establishment of cell lines, and molecular/histopathological analyses and is preservable due to cryoconservation (Scott et al. 2013). Target validation using a broad cohort of clinically relevant PDX models provides more reliable information. As FDA requests drug development to be accompanied by the development of a companion diagnostic test, both require close collaboration between the preclinical experts. Disease-related PDX panels are seen as the optimal basis for the detection of predictive, prognostic, and early-response biomarkers. Possible resistance mechanisms, predictors of response, and rational targets for combinations can be identified, and further the physiological mechanism of action can be analyzed (Amendt et al. 2014).

Next to elementary target or biomarker identification and validation, PDX models will serve as an important tool for the implementation of a personalized medicine.

3 Tumor Models in the Lead Identification and Optimization (LO) Process

The lead identification and optimization is more or less identical with the classical drug development process. Depending on the nature of the target, this will include in vitro assay development, followed by a screening phase of selected compound, peptide, antibody, or RNAi libraries to identify a lead structure. Once a lead structure has been identified, optimization processes are started, frequently in parallel for several leads (Fig. 3).

As the most difficult part of the targeted drug development, this part has to address the molecular mechanism of action in correlation to optimal pharmacodynamic activity (physiological mechanism of action), optimal pharmacokinetics (absorption-distribution-metabolism-excretion (ADME)), toxicity, as well as resistance development.

A large number of functions are getting involved in this integrated preclinical drug development to address:

- The extent of target inhibition in correlation to pharmacological effects (i.e., inhibition of tumor growth, blood flow, metabolism)
- Identification of main indications (primary tumors, metastases)
- Sensitivity on combination with other drugs (drug modifier screen, i.e., high-throughput (HTS) proliferation assays or siRNA technology)
- Sensitivity to drug transporters (ABC transporters), cellular uptake, and intracellular distribution

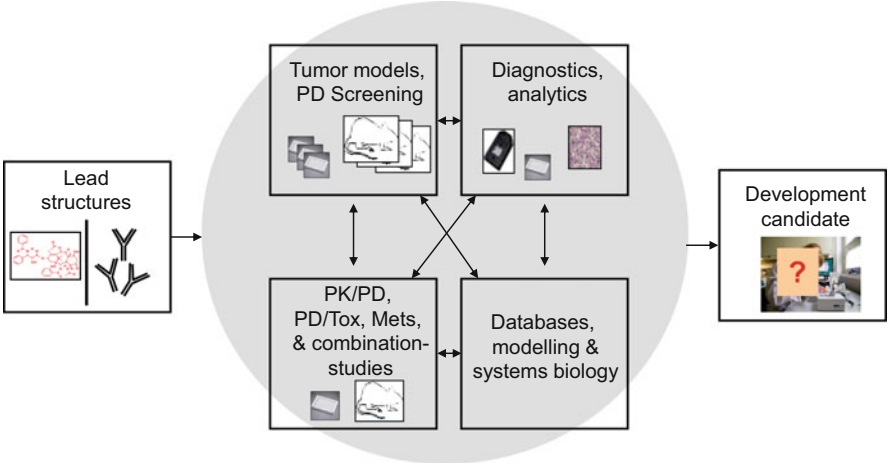

Fig. 3 Integrated research for novel drug candidates. Medicinal chemistry and experimental biology, supported by main preclinical functions, are interactively involved in the preclinical oncology drug development processes

- Gene regulation by the drug in sensitive and resistant models
- Mechanisms of apoptosis and effects on the immune system
- Potential adverse effects and their modulation
- PK/pharmacodynamics (PD) correlations and optimal treatment schedules
- Imaging of response

Similar to the drug development, the biomarker optimization might use or require some of the models in parallel.

In analogy to the TIV process, increased demands on the lead optimization have changed the requests on the disease models. The target-driven approaches now require models with defined levels of target expression which will be mainly generated by genetic modifications and cloning:

- Homogeneous, standardized in vitro tumor models, naturally or genetically engineered with target over- or underexpression for screening (isogenic models), models for classical drug resistance
- Homogeneous, standardized in vivo tumor models, natural or genetically engineered with target over- or underexpression for pharmacodynamic optimization (transgenic mice)
- Models for pharmacokinetic/pharmacodynamic correlation studies in different species (mouse, rat, and/or non-rodent species), models for evaluation of side effects (toxicology) in correspondence to pharmacodynamic effects

4 Translational Research (TR) Process

Translational research in oncology from the perspective of the drug developer should provide the simple answer: "who is the right patient for my new drug," whereas the oncologist is interested in: "which is the right drug for my patient." This means that in the later stages of cancer drug development and in the management of patients with cancer, "predictive biomarkers" are urgently needed which can be used to identify optimal target populations of patients; predict the efficacy of the drug and patient's response, resistance, and toxicity; and rapidly distinguish between nonresponders and patients who respond to therapeutic intervention (Kelloff and Sigman 2012). The major challenge for translational cancer research is the development of patient-specific models conserving the histology and genome of the donor tumors and providing the basis for experimental target validation, individual drug testing, and response prediction. Several cancer system biology consortia are currently developing new patient-derived xenograft (PDX) models. The PDX cultures maintained complex tissue architectures, intra-tumor heterogeneity, driver mutations, and marker expression. Screening of 57 PDX models with 12 compounds revealed pathway-specific drug responses (Rivera et al. 2014). These newly developed PDX models are providing efficient tools for personalized drug development under the given time constraints of clinical settings.

The US Food and Drug Administration (FDA)'s Center for Drug Evaluation and Research (CDER) has provided a guidance document on the qualification process for biomarker (titled "Draft Guidance for Industry: Qualification Process for Drug Development Tools"). Requirements set in this document make clear that the qualification process for a biomarker has many parallels to drug discovery and development, starting with biomarker identification and validation, followed by assay development and optimization, and finally followed by validation in clinical trials. In the preclinical oncology research departments from most pharmaceutical and biotech companies, the translational research has now become an integrative part of the development.

TR needs:

- Large panels of patient-derived tumor models (in vitro and in vivo) representing the heterogeneity of the disease
- Extensive data on the characteristics of these tumor models (gene and protein expression, gene amplifications, mutations, epigenetics, miRNA expression, histology, reference drug sensitivity, and corresponding databases containing all this information and tools allowing bioinformatic analyses)
- Orthotopic models, metastasizing models, and imaging models

This type of research is now frequently performed in academia-industry partnership.

5 Mouse and Rat Strains for Preclinical Oncology Research

Cancer research at bench side has been conducted by the use of animal models for decades. The transplantation of rodent tumor cells into laboratory mice or rats (syngeneic models) has been a widely spread approach for sensitivity testing, biomarker identification, and pharmacokinetic or metastasis studies from the early twentieth century till now. Some of the rodent tumors have been induced by exposure to carcinogens, which led to the development of several specific carcinogen-induced tumor models, i.e., the nitroso-methyl urea (NMU) rat breast cancer model (Shull 2007). For these models, a panel of mice or rat inbred strains have been developed (Table 1).

The observation that athymic nude mice have an impaired immune system, lacking functional T-cells, led to the development of xenotransplantation models. These models allowed for the first time the in vivo growth and passage of human tumors in a laboratory animal (Morton and Houghton 2007). The success of the nude mouse models has intensified the search for further immunodeficient mutations. The discovery of the SCID mutation in mice and crossbreeding with mice bearing the NOD or BEIGE mutation led to the development of further immunodeficient mice with T- and B-cell defects. These mice develop a severe immune deficiency and improved the xenotransplantations. These mice require

Table 1 Commonly used inbred mouse strains (Carter et al. 1952)

Mouse strain	Origin	Features
A	Strong, 1921	Breeding of Kalt-Spring-Harbor and BAGG albinos
BALB	Bagg, 1906	BAGG albino
C3H	Strong, 1920	Breeding of BAGG albino with DBA
C57BL	Little, 1921	Breeding of female 57 with male of Miß Lathrop
CFW	Webster, 1926	"Swiss" breeding of Rockefeller Institute
DBA	Little, 1909	Diluted brown; strains: DBA/1, DBA/2
SWR	Lynch, 1926	Swiss mouse

Table 2 Immune deficient mouse strains (Zhou et al. 2014)

Mouse strain	Features
Nude	T-cell$^-$ B-cell$^+$ NK$^+$
SCID	T-cell$^-$ B-cell$^-$ NK$^+$
NOD/SCID	T-cell$^-$ B-cell$^-$ NK$^{(+)}$, short lifespan
NSB	T-cell$^-$ B-cell$^-$ NK$^{(-/+)}$, short lifespan
NSG	T-cell$^-$ B-cell$^-$ NK$^{(-)}$, long lifespan, no human MHC or cytokines
NOG	T-cell$^-$ B-cell$^-$ NK$^{(-)}$, long lifespan, no human MHC or cytokines
RB	T-cell$^-$ B-cell$^-$ NK$^{(-)}$, long lifespan, no human MHC or cytokines

SCID severe combined immunodeficiency, *NOD* nonobese diabetic, *NK* natural killer cells, *MHC* major histocompatibility complex, *RB* H-2^d RAG2$^{-/-}$ IL-2rgc$^{-/-}$, *NOG* NOD-SCID-IL2rg$^{-/-\ (tm1Sug)}$, *NSB* NOD-SCID-B2m$^{-/-}$, *NSG* NOD-SCID-IL2rg$^{-/-(tm1Unc-J)}$

special standards for breeding and housing which cannot be discussed in this review (Bosma and Carroll 1991).

Actually, the need for more humanized mouse models led to the development of novel genetically modified mice optimized for xenogeneic investigations (summarized in Table 2) (Zhou et al. 2014).

Besides the humanization of mice, another proceeding has been the genetic intervention (knockdown or overexpression of certain genes) to generate transgenic rodents developing spontaneous tumors (Smith and Muller 2013) or using Sleeping Beauty (SB) transposon technology for insertional mutagenesis screening (Tschida et al. 2014). Genetically engineered mice (GEM) are substantial tools for drug development and preclinical "investigations" (Rappaport and Johnson 2014; Lee 2014). Despite the importance of such models for basic oncology research, we will focus on the prospects of patient-derived xenografts (PDX) and how the combination of patient tumor material and humanized mouse strains reaches almost completely the clinical situation.

Even if mice are the more customary laboratory animals, there are several studies using transgenic or transplanted rat models for drug sensitivity testing, biodistribution, or pharmacodynamic experiments providing comparably significant and excellent conclusions in cancer research issues (Lee et al. 2014; Tran et al. 2014; Cao et al. 2014; Zhang et al. 2013; McCullough et al. 2013). Although the genome of rats is identified and manipulable, they have the practical

disadvantage regarding cost and time economy due to their larger size and generation time when compared to mice. For some specific approaches (e.g., physiological/anatomic and hormone-related questions, experimental handling, need of organ material, and PK studies), the laboratory rat might be the better option.

6 Humanized Mice

A current challenge is to overcome the limitations of "classical" mouse xenograft models: the lack of a functional immune background as well as the replacement of the human neoplastic microenvironment by mouse stromal components. Tumor-stroma interactions and blockade of the immune response are well-known factors promoting the tumor growth (Goubran et al. 2014; Wang et al. 2014). The development of appropriate models for the preclinical evaluation of approaches targeting these mechanisms is the focus of tumor model developers. Especially cancer immunologists are limited in their research to immune competent mice with syngeneic tumor models. Given differences in the homology between human and murine proteins or pathways are making the development difficult and less predictive.

Recent approaches have been based on the cotransplantation of human cancer cells as well as human immune or stromal cells into an immunodeficient mouse. As an example, hematopoietic stem cells, engineered NK and T-cells, or mononuclear cells from the peripheral blood have been successfully engrafted on tumor-bearing NOD/SCID mice and used for the evaluation of T-cell-activating therapies (Zhou et al. 2014; Brischwein et al. 2006; Schlereth et al. 2005; Dreier et al. 2003; Fu et al. 2014; Wege et al. 2014; Thibaudeau et al. 2014; Rongvaux et al. 2014; Alcantar-Orozco et al. 2013). Several groups are working on the generation of humanized mouse models for oncological purposes. Fu et al. (2014) established a humanized ovarian tumor stroma due to the transplantation of normal ovarian tissues. The development of a human immune system after implantation of hematopoietic stem cells for a breast cancer xenograft model was described by Wege et al. (2014). The injection of $CD34^+$ cells into specific genetically modified mice results in the production of functional human monocytes, macrophages, and NK cells (Rongvaux et al. 2014).

In summary, xenotransplantation of patient tumors on "increasingly humanized mice" will strongly support predictive preclinical oncology research and moreover provide a fundamental basis for "personalized medicine."

7 Scopes of Patient-Derived Xenografts

Classical cell cultures lack the cellular interactions and structural properties of their donor tissues divesting spatial in vivo-like organization and intra-tumor heterogeneity. This frequently results in different gene expression profiles and drug response readouts (Cree et al. 2010). To better mimic the tumor's composition, in vivo PDX

models for various solid tumor entities have been established and studied with increasing intensity. Unsurprisingly, gene expression and proteome profiles of PDX differ considerably from their respective cell culture counterparts, as PDX rather resembles the genome of the donor tumor (Garralda et al. 2014; Monsma et al. 2012), as shown for ovarian- and colorectal cancer samples. This similarity allows an improved prediction of drug response in efficacy studies. The increase in predictive power prompted us to exploit PDX for accelerating early phase drug discovery. In the past years, preclinical studies using large panels of patient-derived xenograft (PDX) models grown in immunodeficient mice have demonstrated their predictive value for drug and biomarker development (Malaney et al. 2014). Although these PDX models are highly recognized as preclinical research tools, they require substantial resources.

Are preclinical models able to predict the results of clinical trials? Novel strategies to generate more predictive preclinical data for optimization of the clinical development are urgently needed. Appropriate animal models with close similarity to human biology can assure a higher predictability of preclinical studies.

In addition to the general question in which patient population of the drug should be tested (topic of the companion diagnostics development), three further questions have to be addressed by preclinical studies. Is the drug active in metastatic disease, in relapsed tumors, and in the frequency and mechanisms of treatment resistance (Decaudin 2011; Siolas and Hannon 2013)?

Although s.c. xenotransplanted tumors on mice have either a low metastatic potential or not sufficient time to metastasize as the fast growth of the primary tumor requires termination, several approaches have been developed to study metastasis. Most close the clinical metastasis process is modeled by orthotopic tumor transplantation, followed by either lymphogenic or hematogenic metastasis to the lymph nodes, lungs, liver, or peritoneum. Examples have been published for breast cancer (Wenzel et al. 2010), prostate cancer (Park et al. 2010), and lung cancer (Hoffmann et al. 2014). Evaluation of metastasis can be done either by counting visible organ metastases, evaluation of micrometastases by immunohistochemistry, or by human-specific PCR allowing detection of dormant tumor cells (Becker et al. 2002). This procedure is sometimes restricted by the fast growth of the primary tumor. The surgical removal of the primary tumor can permit a longer observation for tumor metastasis.

Given the mentioned limits to model the “natural” metastasis, several surrogate models have been developed. These approaches are using the intravenous, intracardial, or intraperitoneal injection of tumor cells to induce dissemination. This technology has been used successfully to model bone metastasis of breast cancer (Strube et al. 2009). As none of the known tumor models metastasize to the brain, we have developed a surrogate model by implanting either breast or lung tumor cells in the brain, simulating brain metastases (Hoffmann et al. 2009) and allowing the evaluation of drug activity.

Although many tumors initially respond well to the treatment, growth relapse is seen in the majority of patients. Preclinical information, whether a tumor would respond to re-treatment after initial response, is of utmost interest for the clinicians.

Using colon cancer PDX models, it has been recently demonstrated that this can also be simulated in this model system. While initial treatment resulted in tumor regression, a regrowth was observed shortly after treatment suspension. Further treatment cycles were able to re-induce tumor regression by a combination treatment, whereas the single treatments failed to demonstrate activity in recurrent tumors (Schmieder et al. 2014).

These methods can also be used to address the questions regarding resistance mechanisms. Development of second-line resistance to anticancer therapies can be induced due to sustained treatment over several generations of a xenografted tumor. The developed resistant tumors can be used to analyze mechanisms of resistance. Models for antiestrogen-resistant breast cancer have been developed, and by comparing gene expression of the parental and the resistant tumors, Her-2 upregulation has been identified as resistance mechanism (Sommer et al. 2003).

Human tumors accumulate genetic and molecular abnormalities, leading to broad heterogeneity. Large panels of molecular-defined and characterized PDX models reflecting tumor heterogeneity have increased impact for predicting the response to new therapeutic agents in the clinic. The reproducibility, renewability, and availability of tumor material are undisputed advantages.

8 Translational Preclinical Studies with PDX Can Identify Predictive Response Marker

Interesting data have been generated in a study using a large set of patient-derived NSCLC xenograft models (Fichtner et al. 2008). In this panel of NSCLC models, heterogeneous response to Sagopilone treatment was determined in an integrative preclinical phase II study (Hammer et al. 2010). Genome-wide gene expression analysis and mutation analysis of selected genes were used to identify potential markers of response and refractoriness and to explore the mechanism of Sagopilone's antitumor activity in vivo. Overexpression of marker genes (e.g., CA9, CA12, EPHA4, ITGA6) together with TP53 gene expression and mutation has been identified as potential predictive marker for response to Sagopilone (Hammer et al. 2010).

A large panel of colorectal cancer PDX models was developed and tested for drug sensitivity in parallel with a streamlined genetic characterization utilizing panel sequencing and gene expression. The study was used to evaluate to what extend PDX model-based technologies can support translational cancer research processes and even replace clinical experiments (Pechanska et al. 2013; Henderson et al. 2014). In this study it has been confirmed that kRas mutations are a strong predictor for resistance to cetuximab (with 86% specificity), and in addition mutations in bRaf and PI3K have been identified as additional predictive biomarker for drug response (Pechanska et al. 2013).

PDX of pancreatic cancer has been also used as a model for translational medicine (Behrens et al. 2014). For pancreatic cancer, similarity between the activity of gemcitabine in PDX models and respective clinical trial data is notable (Garrido-Laguna et al. 2011). Further, PDX can be utilized as a potential screening

platform for clinical trials as it could be shown in a prospective study that the combination of *nab*-paclitaxel and gemcitabine is effective in pancreatic PDX. This outcome is correlated with the clinical efficacy of the combination. Indeed, in a randomized phase III study, this regime has shown to provide a survival benefit for patients with advanced pancreatic cancer, and it is likely to become a standard of care in this setting (Von Hoff et al. 2013).

In another PDX study with gemcitabine, the expression of gemcitabine-activating enzyme deoxycytidine kinase was identified as a predictor of drug efficacy. A subsequent analysis of this marker in clinical samples confirmed these results (Rubio-Viqueira et al. 2006; Sebastiani et al. 2006).

9 Current Limitations

One disadvantage of PDX is the loss of the human tumor microenvironment during engraftment of patient material. This may affect tumor progression and is discussed as one reason for the low take rate of breast and prostate xenografts (Hidalgo et al. 2014). A review of Fang and DeClerk (2013) showed clearly the impact and benefit of the tumor microenvironment as target for anticancer treatment. Several integrin inhibitors (EMD 121974, CNTO 95, MEDI-522) that impair the communication between tumor cells and extracellular matrix are under clinical investigation (Dechantsreiter et al. 1999; O'Day et al. 2011; Hersey et al. 2010). The therapeutic potential of the tumor surrounding tissue is discussed for several entities, like pancreatic (Rossi et al. 2014), breast (Nwabo Kamdje et al. 2014), and prostate cancer (Chiarugi et al. 2014). However, after xenotransplantation, the human stromal components are replaced by a murine texture within 3–9 weeks (Hylander et al. 2013). With respect to therapeutic approaches targeting the human tumor microenvironment, the classical PDX models are therefore less feasible. The stroma replacement can be decelerated by the engraftment of large, non-disrupted tissue fragments and by the use of NOD/SCID mice with knockdown of IL2Ry (Bankert et al. 2001, 2011). In addition, the cotransplantation of human fibroblasts has been evaluated to generate PDX models with a more "humanized" microenvironment (Hoffmann unpublished results).

As mentioned earlier, a human microenvironment is strongly needed for another pillar of tumor therapy – the activation of immune reactions. Xenotransplanted human tumor cells are growing well in immunodeficient mice, very similar to the patient where they have escaped the body's immune control. Whereas in the patient a functional immune system is present and can be redirected against the tumor cells, the currently used immunodeficient mouse strains are mainly lacking functional immune cells (i.e., tumor-associated macrophages, dendritic cells, cytotoxic T-cells) and secretion of inflammatory cytokines (Fang and DeClerk 2013; Duechler et al. 2014; daChuna et al. 2014; Paulsson et al. 2014). Consequently, the preclinical evaluation of immunotherapeutic strategies in PDX has certain limitations. Therefore, the less predictive syngeneic mouse models or the cotransplantation of human peripheral blood mononuclear cells has been the

standard for the evaluation of immunological therapies. In the last years, transgenic mice stimulating the differentiation of cotransplanted human hematopoietic stem cells have been developed. These mice will develop a functional human immune system, allowing the analysis of new immune therapies (Alcantar-Orozco et al. 2013; Cook et al. 2013; Futakuchi and Singh 2013; Reisfeld 2013; Stromnes et al. 2014).

The establishment and maintenance of patient-derived xenografts are time and cost intensive. Depending on the tumor type, engraftment rate of P1-generation ranges from 15% to 80% and usually takes 2–3 months. The following conservation, characterization, and validation process need further 4–6 months setting the time lines between 6 and 9 months.

These time lines are challenging for the use of the PDX for individual drug response prediction studies. Data will not be available for first-line treatments; however, it could be of valuable help for planning second-line therapies after tumors relapsed.

Working with in vivo tumor models set high demands on the qualification of the scientific personal and the laboratories (clean room, biobanking equipment, and molecular biology).

10 Outlook

Depending on the stage of the drug discovery program, different models are required. For primary in vitro screening, cell lines can be utilized easily from the available large panels or generated by genetic engineering. They can be selected based on the target or the question to be answered. For secondary in vitro screening, larger panels of tumor cell lines with known sensitivity or resistance to available standard drugs are used for further profiling.

Classical 2D cell cultures lack the cellular interactions and structural properties of their donor tissues divesting spatial in vivo-like organization and intra-tumor heterogeneity. This frequently results in different gene expression profiles and drug response readouts. To better mimic the tumor's composition, in vitro 3D models for various solid tumor entities have been established and currently studied with increasing intensity.

Often a differential pattern of sensitivity can be observed using in vivo models. This gap between in vitro and in vivo activity constrains that in vivo experiments are still crucial and remain an integral part to evaluate tumor response in the near future.

Although mouse xenograft models derived from established human cancer cell lines have undoubtedly enhanced the understanding of the antitumor activity of novel anticancer agents, these models have several disadvantages. Depending on the number of cell passages, xenografts can behave very differently to the primary tumor (Haddad and Yee 2008), and combined with other deficiencies in preclinical approaches (Sharpless and Depinho 2006), this can reduce the relevance of established xenograft models for predicting the probability of success of anticancer

drugs in clinical studies for some tumor localizations. Analysis of antitumor activity in patient-derived xenograft (PDX) models has provided a more accurate selection process for the identification of agents which have activity in clinical trials, suggesting that some of these models may provide a useful hint for activity in the clinic (Furman et al. 1999). A fundamental move for the improvement of PDX is the humanization of these models. Different approaches such as the establishment of a human stroma, the cotransplantation of human hematopoietic stem cells, or the development of humanized homing niches have been successfully realized (Fu et al. 2014; Wege et al. 2014; Thibaudeau et al. 2014). Another effective method is the generation of novel mouse strains with humanized setting dropping highly informative preclinical data (Zhou et al. 2014; Rongvaux et al. 2014).

Genome-wide analyses of gene expression using oligonucleotide microarrays have allowed the determination of molecular characteristics present in xenograft models that mirror tumor behavior and relate to disease progression and survival (Nevins et al. 2003). Furthermore, correlations between the growth of xenograft models derived directly from patient tumors and the clinical prognosis of donor patients have been reported (Angevin et al. 1999; Peterson and Houghton 2004). In the future, the use of patient-derived human tumor xenografts will therefore play a key role in the search for more efficacious cancer treatments (Perez-Soler et al. 2006; Fichtner et al. 2004, 2008; Becker et al. 2004; Garber 2009). The ability to identify and assess antitumor activity in well-characterized xenografts in correlation with particular genetic or molecular characteristics may aid the development of new therapeutic regimens.

Conclusions from what we discussed here are:

- Drug discovery, systems biology, and translational research are moving together to address all the new hallmarks of cancer and increasing the success rate of drug discovery.
- In vitro versus in vivo models or vice versa – both models have limitations and advantages, however, when used critically, all generate important and reliable results.
- Panels of patient-derived xenograft (PDX) models represent an important tool for translational research.
- Predictive value of the preclinical models is increasing steadily; however, even genetically engineered “humanized” mice are still not men.

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Translational In Vivo Models for Cardiovascular Diseases

Daniela Fliegner, Christoph Gerdes, Jörg Meding, and Johannes-Peter Stasch

Contents

Abstract

Cardiovascular diseases are still the first leading cause of death and morbidity in developed countries. Experimental cardiology research and preclinical drug development in cardiology call for appropriate and especially clinically relevant in vitro and in vivo studies. The use of animal models has contributed to expand our knowledge and our understanding of the underlying mechanisms and accordingly provided new approaches focused on the improvement of diagnostic and treatment strategies of various cardiac pathologies.

Numerous animal models in different species as well as in small and large animals have been developed to address cardiovascular complications, including heart failure, pulmonary hypertension, and thrombotic diseases. However, a perfect model of heart failure or other indications that reproduces every aspect

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of the natural disease does not exist. The complexity and heterogeneity of cardiac diseases plus the influence of genetic and environmental factors limit to mirror a particular disease with a single experimental model.

Thus, drug development in the field of cardiology is not only very challenging but also inspiring; therefore animal models should be selected that reflect as best as possible the disease being investigated. Given the wide range of animal models, reflecting critical features of the human pathophysiology available nowadays increases the likelihood of the translation to the patients. Furthermore, this knowledge and the increase of the predictive value of preclinical models help us to find more efficient and reliable solutions as well as better and innovative treatment strategies for cardiovascular diseases.

Keywords

Animal models, Heart failure, Preclinical cardiology, Pulmonary hypertension, Thrombotic diseases, Translational research

1 Introduction

Although drugs are mainly optimized and developed for the treatment of human diseases, the first testing of their efficacy and safety has to occur in animal studies. Without a proven efficacy in preclinical animal models, ideally mirroring many aspects of the human pathophysiology, and additional extensive safety studies, no allowance for first-in-man studies will be granted by the authorities.

Translational animal models are, therefore, the base of the successful development of innovative drugs in all indication fields. Predictability for clinical trials with patients is the key and the most challenging part of the development and use of animal models. There are species differences between, e.g., signaling cascades, receptor sensitivities, receptor subtypes, and the pharmacodynamic and pharmacokinetic qualities of a new drug. Drug development becomes even more challenging when factors such as stage of disease, progression of disease, comorbidities, and other risk factors as a predisposition, genetic polymorphism, or epigenetics have to be taken into account. Regarding safety aspects, findings in one species might be not seen in humans and vice versa. This makes the field of in vivo pharmacology very challenging but also one of the most interesting and exciting in drug development before the first application of a new drug into patients.

Cardiovascular diseases (CVD) are still the leading cause of death and morbidity in developed countries. Cardiac and vascular complications are complex multifactorial pathologies, in which both genetic and environmental factors are implicated, thus making them very difficult to prevent. Due to the complexity and heterogeneity, we will focus in this chapter on three major disease indications, which are heart failure, pulmonary hypertension, and thrombotic diseases.

2 Animal Models in Cardiovascular Diseases: The Demands on Target Identification, Validation Models, and the Translation

2.1 Heart Failure

HF is a complex disorder with heterogeneous etiologies and remains one of the most threatening diseases known. It is a clinical syndrome attributable to a multitude of factors that begins with the compensatory response known as hypertrophy, followed by a decompensated state that finally results in heart failure.

In cardiac research the use of animal models has been conducted for decades. Basic and translational scientists use small animal models to explore and to provide important insights into the pathophysiology of CVD including HF and to develop novel treatment strategies to prevent end-organ damage and slow down the progression of the fatal disease. Thus, research and preclinical drug discovery depend highly on specific in vivo disease models mirroring the human pathophysiology.

Therefore, effective animal models as well as clinically relevant readout parameters are essential for preclinical testing of drugs to get first hints for in vivo efficacy in CV diseases. State-of-the-art assessment of cardiac function by using hemodynamics, echocardiographic parameters, high-resolution imaging, assessment of relevant biomarker, and molecular characterization (such as gene and protein expression/activity, histology) in these animal models is mandatory to categorize, quantify, and monitor drug efficacy on cardiac disease progression and improvement in animal models.

HF has been induced in different species with volume overload, pressure overload, fast pacing, myocardial infarction, or cardiotoxic drugs. Models of genetically induced cardiomyopathy are also available in small animals. Here, we will summarize some of the most common and translational rodent models of HF, designed and used to answer and cover some aspects related to human pathologies.

2.2 HF Induced by Myocardial Infarction

Myocardial ischemia or infarction (MI) is a common risk factor for the development of congestive heart failure in humans. MI is associated with left ventricular remodeling including changes in geometry, structure, and function. Persistence of the remodeling, although initially adaptive, ultimately precipitates the progression of heart failure. The mechanism responsible for this deleterious transition from adaptive remodeling to dysfunction is not fully understood and is more likely under the influence of the neurohormonal axis, loading conditions of the remaining myocardium, and alteration of the extracellular matrix (Feldman et al. 2001; Francis and Wilson Tang 2003). In an effort to reproduce the progression of HF in humans with coronary disease more closely, models of myocardial ischemia have been developed with different techniques.

The chronic occlusion of the left anterior descending coronary artery (LAD) has been used extensively to induce myocardial ischemia and postinfarction disturbance in different animal species. Functional parameters, such as left ventricular end-diastolic pressure (LVEDP), cardiac output, or the first derivative of pressure of time (dp/dt max), are assessed in this model. The non-infarcted myocardium maintained normal blood flow, while the blood flow in the infarcted myocardium was significantly reduced to normal (Pfeffer and Braunwald 1990). This is an essential model for a subset of patients in whom postinfarction dilated cardiomyopathy developed after a single, moderate-sized infarct. Since there are no confounding variables (arteriosclerosis, several infarcts, hypertension, diabetes, etc.), this model provides an opportunity to understand the progression of a normally perfused and contractile myocardium to a hypocontractile status after transmural infarction.

The Pfeffer group was the first who used this model and observed that the angiotensin-converting enzyme inhibitor "captopril" therapy reduced LV chamber dilation, improved LV systolic function, and increased survival in rats with moderate or large MIs (Pfeffer and Braunwald 1990). This groundbreaking work in the rat MI model led to clinical trials testing the utility of the angiotensin-converting enzyme inhibitor, captopril, in post-MI patients with reduced LV function. A multicenter Survival and Ventricular Enlargement (SAVE) trial was conducted in which captopril or placebo was administered 3–16 days following MI in patients with reduced LV function. Captopril decreased all-cause mortality by 19% with a 22% reduction in heart failure hospitalizations after a mean follow-up period of 42 months; the reductions in mortality and morbidity were associated with less LV dilation or remodeling during the first year of therapy (Pfeffer et al. 1985). This study demonstrated very elegantly the utility of small animal models to explore new and potentially important therapies for heart failure. The rat MI model was also essential in establishing the beneficial effects of angiotensin II type 1 receptor antagonists (ARAs) on LV structure and function following MI (Pfeffer et al. 1985).

2.3 HF Induced by DOCA

The treatment with deoxycorticosterone acetate (DOCA)-salt, a synthetic mineralocorticoid derivative, is widely used to induce HF mediated by volume overload in several species and conditions and reviewed in detail by Iyer et al. (2010). The DOCA-salt model results in hypertension via salt retention and an increase in circulating blood volumes (Gavras et al. 1975). These changes are also accompanied with the development of cardiac hypertrophy, fibrosis, conduction abnormalities, and endothelial dysfunction, followed by cardiovascular remodeling. Furthermore, the DOCA-salt hypertensive rat is a model for human primary aldosteronism and is associated with a markedly depressed renin-angiotensin system and thus has been regarded as an angiotensin-independent model with significantly decreased circulating plasma renin activity. Similar characteristics have been made in patients with hypertension and heart failure.

This animal model is quite robust to predict blood lowering capabilities of antihypertensive agents in patients.

Recently, Kolkhof et al. investigated the tissue distribution and chronic cardiorenal end-organ protection of finerenone, a nonsteroidal MR antagonist (MRA), in comparison to the steroidal MRA eplerenone in different preclinical rat disease models (Kolkhof et al. 2014). Finerenone treatment prevented in DOCA-salt challenged rats from functional as well as structural heart and kidney damage at dosages not reducing systemic blood pressure. Furthermore, finerenone reduced cardiac hypertrophy, plasma prohormone of brain natriuretic peptide (pro-BNP), and proteinuria more efficiently than eplerenone when comparing equi-natriuretic (i.e., an indirect measure of anti-kaliuretic) doses. Based on these preclinical investigations, finerenone may offer end-organ protection with a reduced risk of electrolyte disturbances compared with steroidal MRAs in patients with chronic heart and kidney diseases (Bauersachs 2013). Accordingly, finerenone was investigated in a multicenter, randomized, double-blind, placebo-controlled, parallel-group clinical phase II study called "ARTS" (MinerAlocorticoid Receptor Antagonist Tolerability Study) among patients with heart failure with reduced left ventricular ejection fraction (HFrEF) and chronic kidney disease (Pitt et al. 2012). In these patients, 5 and 10 mg/day of finerenone was at least as effective as spironolactone 25 or 50 mg/day in decreasing BNP, NT-pro-BNP, and urinary albumin, but it was associated with lower increases in serum potassium, lower incidences of hyperkalemia, and worsening of renal function (Pitt et al. 2013).

In summary, the novel nonsteroidal MRA finerenone demonstrated higher efficacy with respect to end-organ protection when comparing equi-efficient natriuretic (anti-kaliuretic) doses of a steroidal MRA in chronic preclinical models of heart failure. Finerenone demonstrated comparable efficacy to a steroidal MRA in patients with HFrEF and CKD with significantly lower incidences of dangerous hyperkalemia and renal failure.

2.4 Pulmonary Hypertension

Pulmonary hypertension (PH) is a severe, progressive, life-changing and life-threatening disorder of the heart and lungs, characterized by increased blood pressure in the pulmonary arteries (PAs) that can lead to heart failure and death. Patients with PH develop a markedly decreased exercise capacity and a reduced quality of life. The most common symptoms of PH include shortness of breath, fatigue, dizziness, and fainting, all of which are worsened by exercise. PH is clinically categorized into five groups depending on clinical presentation, etiology, and therapeutic approach (Simonneau et al. 2013; Galiè et al. 2009). Early diagnosis and accurate identification of the PH group are essential, as a delay in treatment initiation can have a negative impact on survival. Continuous treatment monitoring is then vital to ensure that patients are receiving optimal care for their particular group and stage of disease.

Pulmonary blood pressure can be increased on the precapillary side of the lung in PH Groups 1 (pulmonary arterial hypertension [PAH]), 3 (PH due to lung diseases and/or hypoxia), 4 (chronic thromboembolic pulmonary hypertension [CTEPH]), and 5 (PH with unclear multifactorial mechanisms); postcapillary hypertension corresponds to Group 2 (PH due to left heart disease) (Simonneau et al. 2013; Galiè et al. 2009). In patients, PH can be diagnosed and quantified by right heart catheterization, which also discriminates between post- and precapillary PH. Secondary to the increased PA blood pressure, exercise capacity in patients with PH is significantly decreased, and due to the pressure overload of the right heart induced by increased pulmonary vascular resistance (PVR), morphological changes of the right heart occur that may lead to right heart failure.

Effective animal models and corresponding readout parameters are essential to test PH drugs in the preclinical setting. Exercise capacity, accurate hemodynamic measurements, and advanced imaging tools are necessary to correctly categorize, quantify, and monitor disease progression and regression of PH in animal models. Exercise capacity can be assessed using treadmill testing. However, there are multiple determinants of maximal exercise capacity, including right and left ventricular function, pulmonary function, skeletal muscle function, and activity of the peripheral and central nervous systems. A complete hemodynamic assessment is needed to fully understand PH animal models, including PVR measurement and left and right heart catheterization to exclude PH Group 2. In rodent and other animal models used to study PH, the normal PA pressure is the same as that in healthy humans. However, in rats and mice the heart rate is 5–10 times faster than in humans. Pulmonary hemodynamics are optimally measured in lightly anesthetized, closed-chested animals. Although cannulating the PA is faster and requires less expertise in the open-chest preparation, opening the thorax falsely reduces cardiac output, heart rate, and PA pressures (particularly end-diastolic pressures). Imaging and even longitudinal imaging of the heart to follow morphological and functional changes in animal models can be performed with the same modalities used routinely in clinics, such as ultrasound imaging, magnetic resonance imaging (MRI), or computed tomography (CT). In addition, after dissection of the animal at the end of the study, the right and left ventricle weight can be measured to determine the hypertrophy of the right heart (Ryan et al. 2009).

Recently, the soluble guanylate cyclase (sGC) stimulator riociguat has been approved to treat PH Groups 1 (PAH) and 3 (CTEPH), thereby providing a novel approach for the treatment of different forms of PH (Ghofrani et al. 2013a,b). An important part of the development program for riociguat included a broad in vitro and in vivo pharmacologic characterization of this new pharmacologic principle; sGC stimulation was performed, including hemodynamic studies in different animal models, long-term studies in experimental cardiovascular diseases models, and studies in the target indication PH. sGC stimulators have shown beneficial effects in animal models with different underlying pathologies of acute and chronic PH. Improvements in clinically relevant parameters such as decreased pulmonary blood pressure as well as remodeling of the heart and vessels have been demonstrated. These animal models include ischemia-reperfusion injury-induced

pulmonary vascular dysfunction in isolated rabbit lungs, acute pulmonary embolism in dogs, acute PH in lambs, monocrotaline (MCT)-induced PH in rats, and chronic hypoxia-induced PH in mice (Stasch and Hobbs 2009; Evgenov et al. 2006; Stasch and Evgenov 2013). The effects of riociguat in these PH animal models are outlined below.

In a mouse model of chronic hypoxia-induced PH, treatment with Riociguat significantly reduced right ventricular systolic pressure (RVSP), ventricular hypertrophy, myocardial fibrosis, and structural remodeling of the lung vasculature (Schermuly et al. 2008). In a rat model of severe PH induced by MCT, which presents with a marked increase in right ventricular systolic pressure, total pulmonary vascular resistance (TPR) and right heart hypertrophy. Riociguat significantly decreased RVSP, TPR, and right heart hypertrophy without a change in systemic arterial pressure (Schermuly et al. 2008). In addition, chronic treatment with riociguat in this rat model resulted in a significant reduction of fully muscularized PAs and increased the percentage of non-muscularized PAs. In another rat model with severe angioproliferative PAH, riociguat significantly decreased RVSP and right ventricular hypertrophy, increased cardiac output, and decreased total pulmonary resistance, compared with vehicle (Lang et al. 2012). Riociguat also significantly decreased the right ventricular collagen content, improved right ventricular function, and significantly lowered the proportion of occluded arteries compared with animals receiving vehicle. In another study, systemic blood pressure increase in a therapeutically relevant low NO/high renin heart failure model (L-NAME-treated renin transgenic rats) was completely prevented and survival was improved by administration of riociguat. Riociguat also reduced cardiac and renal end-organ damage as indicated by lower plasma atrial natriuretic peptide and plasma creatinine and urea levels, respectively, and lowered relative left ventricular weight, cardiac interstitial fibrosis, glomerulosclerosis, and renal interstitial fibrosis. Finally, in salt-sensitive Dahl rats, riociguat markedly attenuated systemic hypertension, improved systolic heart function, increased survival, and ameliorated fibrotic tissue remodeling and degeneration in the heart and kidneys (Stasch and Evgenov 2013).

In phase III clinical trials, riociguat significantly improved pulmonary vascular hemodynamics and increased exercise capacity in patients with PAH and CTEPH (Ghofrani et al. 2013a,b). Furthermore in smaller clinical trials, riociguat improved cardiac output and PVR in patients with PH associated with interstitial lung disease, reduced mean pulmonary arterial pressure (mPAP) and PVR in patients with PH associated with chronic obstructive pulmonary disease, and improved cardiac index and PVR in patients with PH associated with left ventricular dysfunction (Stasch and Evgenov 2013; Bonderman et al. 2013). These promising results suggest that sGC stimulators may constitute a valuable new therapy for PH. Other trials of riociguat are in progress, including long-term extensions of the phase III trials investigating the efficacy and safety of riociguat in patients with PAH and CTEPH (Rubin et al. 2015; Simonneau et al. 2015). Finally, sGC stimulators may also have potential therapeutic applications in other diseases, including heart failure, lung fibrosis, scleroderma, and sickle cell disease.

In summary, rodent PH animal models have been highly predictive for clinical trials with PH patients testing sGC stimulators. PVR and the corresponding increased pulmonary blood pressure were significantly decreased in both rodents and patients.

2.5 Thrombotic Diseases

Anticoagulant drugs are effective standards of care for the prevention and treatment of thromboembolic disorders. Among other drawbacks, the use of traditional agents like heparins is limited by their parenteral route of administration or by a significant variability of pharmacodynamic responses requiring routine monitoring and dose adjustments like with vitamin K antagonists (Laux et al. 2009). In addition, anticoagulants with broad effects on multiple coagulation factors display a narrow dose range between antithrombotic efficacy and the risk of increasing the rate of severe bleedings. Demonstrating and identifying this therapeutic window is the most important challenge to preclinical and early clinical models of thrombosis and hemostasis.

The search for selective novel oral anticoagulants (NOACs) has led to the discovery and successful development of direct inhibitors of coagulation factor Xa (FXa: rivaroxaban, apixaban; Perzborn et al. 2005; Wong et al. 2008) and of thrombin (FIIa: dabigatran; Wienen et al. 2007). FXa has a key function in blood coagulation because it initiates the final common pathway of coagulation by catalyzing thrombin generation. Thrombin is the central protease in the cascade leading to fibrin clot formation and platelet activation. Biochemical assays on isolated enzymes and on protease complexes as well as functional test systems in plasma and in whole blood provide data on the activity of each step in the coagulation system and the modulation by anticoagulants (Fig. 1).

In contrast to other organ systems, excellent access to the target tissue – the blood – both in experimental animals and humans allows early comparative assessment of compound activity profiles across species. This enables the selection of appropriate laboratory animals for studying effects of drug candidates in models of thrombosis and hemostasis. The FXa inhibitors rivaroxaban (Kubitza et al. 2013) and apixaban (Wong et al. 2011) show distinct in vitro potency profiles between species both biochemically on FXa (Ki) and functionally in plasma (prothrombin time, PT). Species dependency on clotting inhibition with dabigatran has been demonstrated in different coagulation tests (Eisert et al. 2010).

2.6 Animal Models for Anticoagulation Testing

After careful selection of the appropriate animal species and depending on the mode of action and on the intended clinical indication, the following criteria need to be considered from a range of thrombosis models:

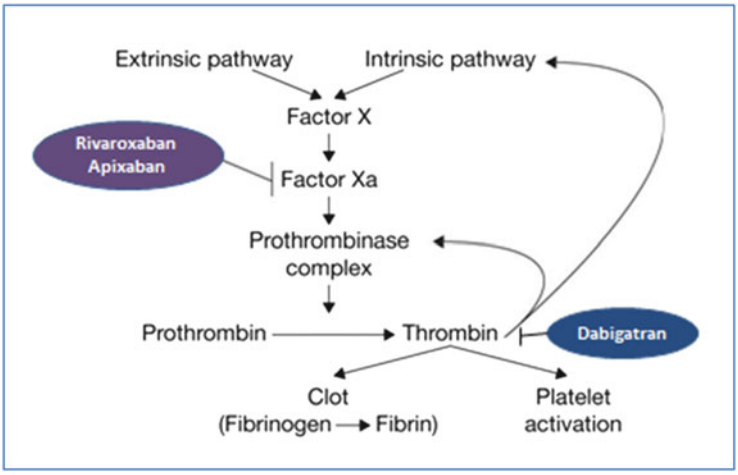

Fig. 1 The blood coagulation pathway addressed by NOACs

- Vessel type (arterial/venous, large vessel/microcirculation, high/low shear)
- Type of injury (mechanical, thermal, chemical, electrical, laser, artificial surface)
- Degree of vessel wall damage (superficial/deep, affected area)
- Readout (thrombus size/weight, flow reduction, occlusion time)
- Timing of dosing and thrombus induction (prophylactic vs. therapeutic setting)

In any case the models of choice need to be well validated with drugs established in the clinical setting. Animal experiments should be monitored by pharmacodynamic (PD) biomarkers suitable for use in later clinical studies. As preparation steps before starting complex thrombosis experiments, orientating pharmacokinetic (PK) and PD/PK studies are recommended to demonstrate consistency with in vitro data and to ensure sufficient compound exposure for antithrombotic efficacy.

Bleeding models are mandatory for the assessment of the therapeutic window. Ideally, the bleeding risk will be evaluated in the identical experiment used for studying antithrombotic efficacy. This combination model approach significantly reduces inter-experiment and inter-individual sources of variability. Confounding effects due to dynamics of compound plasma concentration can be minimized by establishing steady-state plasma levels (i.e., continuous infusion, Wong et al. 2011).

Further considerations for the choice of bleeding test are:

- Species, vessel type (arterial/venous/microvascular)
- Target tissue (skin, mucosa, parenchyma, gastrointestinal tract, brain)
- Type of injury (incision, transection, blunt trauma, collagen digestion)
- Readout (bleeding time (initial/cumulative), rebleedings, blood volume)

- Validation with drugs known to affect bleeding risk in humans

The extrapolation of preclinical results, in particular, bleeding time data, to humans requires caution. Provoked bleeding from peripheral skin locations in anesthetized healthy animals may not directly translate into the risk of clinically relevant hemorrhages in patients. Therefore, the future direction for both bleeding and thrombosis models will aim for better organ specificity (e.g., cerebral, gastrointestinal bleedings), studies on disease backgrounds (e.g., diabetic, atherosclerotic), and experiments on top of co-treatments (e.g., antiplatelet or thrombolytic drugs). Not a single model but rather a range of models carefully selected and designed for the target indication will help to mitigate the risk for later clinical development.

In conclusion, the discovery process for novel antithrombotics is facilitated by a range of well-characterized preclinical models for efficacy and – still to a lesser degree – for safety. Easy access to the target organ throughout all stages of research and development provides the unique opportunity for multiple cross-checks between bench and bedside with the goal of optimizing efficacy of preclinical models.

3 Conclusion and Limitations

Translational animal models are the key for the successful development of new innovative drugs. Novel and optimized drug therapies demand for a deep understanding of the underlying pathophysiology and its underlying mechanism of the specific disease. In vivo models allow identifying the mode of action of a specific target that confers, e.g., cardioprotection, the prevention of disease progression, or the improvement of heart diseases.

Personalized medicine is the upcoming challenge for the future development of innovative drugs. The further development and optimization of existing animal model to mirror the pathophysiology and characteristics of patient subgroups will, therefore, be the next big step to translational animal models fulfilling these upcoming requirements. Most animal models have limitations, reflecting only certain aspects of diseases, and do not fully recapitulate the pathophysiology of the human disease state and the respective disease subgroups. However, animal models are the only option when used critically to generate important and reliable insights into the pathophysiology of CV diseases and the efficacy of new drugs, and this will also not change in the future.

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Pharmacokinetics in Drug Discovery: An Exposure-Centred Approach to Optimising and Predicting Drug Efficacy and Safety

Andreas Reichel and Philip Lienau

Contents

Abstract

The role of pharmacokinetics (PK) in drug discovery is to support the optimisation of the absorption, distribution, metabolism and excretion (ADME) properties of lead compounds with the ultimate goal to attain a clinical candidate which achieves a concentration–time profile in the body that is adequate for the desired efficacy and safety profile. A thorough characterisation of the lead compounds aiming at the identification of the inherent PK liabilities also includes an early generation of PK/PD relationships linking in vitro potency and target exposure/engagement with expression of pharmacological activity (mode-of-action) and efficacy in animal studies. The chapter describes an exposure-centred approach to lead generation, lead optimisation and candidate selection and profiling that focuses on a stepwise generation of an understanding between PK/exposure and PD/efficacy relationships by capturing target exposure or surrogates thereof and cellular mode-of-action readouts in vivo. Once robust PK/PD relationship in animal PD models has been constructed, it is

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translated to anticipate the pharmacologically active plasma concentrations in patients and the human therapeutic dose and dosing schedule which is also based on the prediction of the PK behaviour in human as described herein. The chapter outlines how the level of confidence in the predictions increases with the level of understanding of both the PK and the PK/PD of the new chemical entities (NCE) in relation to the disease hypothesis and the ability to propose safe and efficacious doses and dosing schedules in responsive patient populations. A sound identification of potential drug metabolism and pharmacokinetics (DMPK)-related development risks allows proposing of an effective de-risking strategy for the progression of the project that is able to reduce uncertainties and to increase the probability of success during preclinical and clinical development.

Keywords
ADME · Candidate profiling · Drug discovery · Exposure · Lead generation · Lead optimisation · Pharmacokinetics · PK/PD · Prediction

1 Introduction

Drugs can only exert their desired effects if they are able to bind to the intended target proteins in the body. Although this drug–target engagement is not a guarantee for efficacy, it is a prerequisite for pharmacological effects in the target cells. Efficacy thus is not only dependent on the potency of a drug but also on the exposure of the drug to the pharmacologically active site. Exposure means that the drug must reach the target site at sufficiently high concentrations and for a sufficiently long period of time after it has been administered to the patient. Pharmacokinetics (PK) is the discipline that explores the absorption, distribution, metabolism and excretion (ADME) behaviour of drugs which are the processes that control the kinetics of the concentration–time profile in the blood circulation and the body tissues and organs.

Traditional dose–response concepts are insufficient for the understanding of drug effects if the body and target tissue exposure of the drug is not being considered (Figs. 1 and 2). In a retrospective analysis of Phase II clinical trials, Morgan et al. (2012) extracted three "pillars of survival" for clinical proof-of-concept studies in patients: demonstration of (1) drug–target exposure, (2) drug–target binding and (3) expression of pharmacological activity. The authors concluded that an integrated understanding of the fundamentals of the pharmacokinetic (PK) and pharmacodynamic (PD) principles of a drug is a key success factor with preclinical experimental evidence for at least two of the three pillars significantly enhancing the success rate of drug discovery programmes during clinical development. The conclusions of this analysis have recently been further supported and expanded by a retrospective analysis of AstraZeneca's project portfolio (Cook et al. 2014).

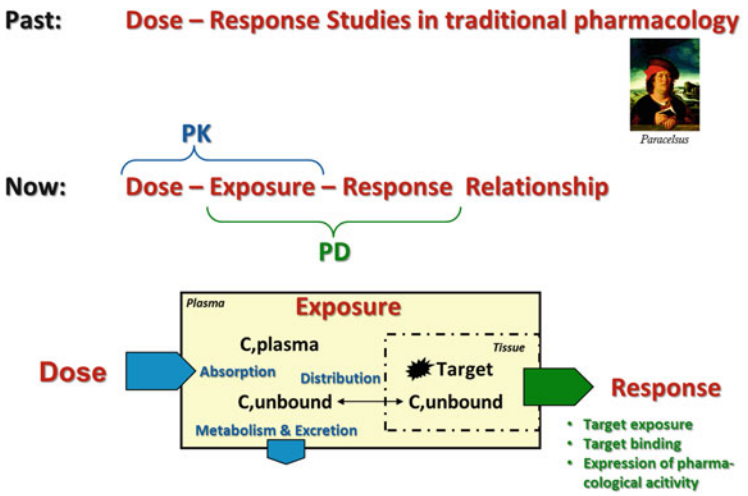

Fig. 1 Schematic illustration contrasting the past dose–response paradigm with the concept of dose–exposure–response relationships incorporating pharmacokinetic (PK) processes controlling the concentrations in the body (*blue*) and the processes required to elicit a pharmacological response (*green*), with the unbound plasma and (target) tissue concentrations representing the exposure link between dose and response

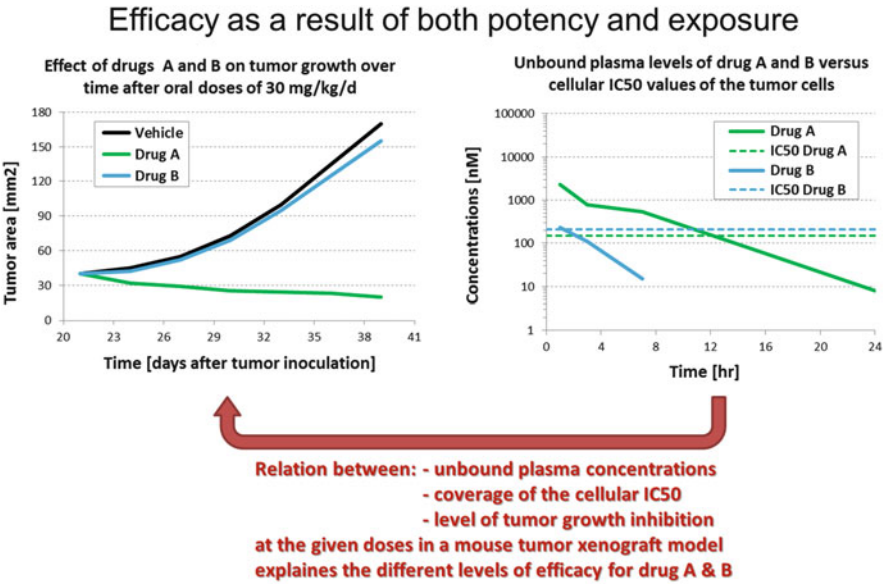

Fig. 2 Illustration of the impact of target exposure on efficacy. Drug A and B which are almost equipotent (unbound cellular IC50s of 150 and 210 nM, respectively) have been applied daily at the same dose in a tumour xenograft study in mice. The inefficacy of Drug B can be explained by the unfavourable relation between the unbound plasma concentration vs. IC50, while the strong tumour growth inhibition (TGI) effect of Drug A is in accordance with the target coverage of the unbound concentrations

Addressing exposure aspects right from the start of a new project and throughout all phases of the drug discovery process has a significant impact on the selection and optimisation of compounds and the prospect to turn them into viable drug candidates for preclinical and clinical development. The "free drug hypothesis" (Smith et al. 2010) is fundamental to this concept.

The wide acceptance that efficacy (and safety) is not only a result of the potency of a drug at the target (and off-target) protein, but also depends on the exposure of and the engagement with these target proteins has secured pharmacokinetics an immanent role in the drug discovery process. More than a decade since PK representatives have become integral part of drug discovery projects, the attrition rate of projects during clinical development due to PK liabilities went down significantly from originally 40% to less than 10% today (Kennedy 1997; Frank and Hargreaves 2003; Empfield and Leeson 2010; DiMasi et al. 2010, 2013). The attrition at the point of transition to preclinical development is even less allowing viable projects to progress from the discovery to the development phase with a higher probability of success.

This chapter describes how pharmacokinetics supports drug discovery based on an exposure-centred approach by identifying and optimising those PK liabilities which enable both the efficacy and safety of drug candidates and by designing in those ADME properties that result in an adequate PK profile. The role of pharmacokinetics in the different phases of drug discovery is outlined in Fig. 3 which also serves to structure the chapter.

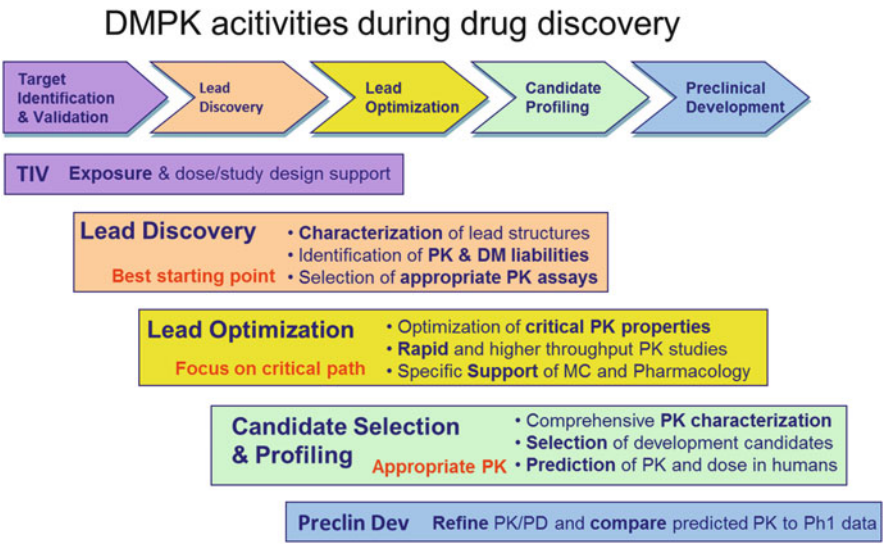

Fig. 3 Overview of the main tasks of DMPK support during the different phases of drug discovery. *TIV* target identification and validation, *PK* pharmacokinetics, *DM* drug metabolism, *MC* medicinal chemistry

2 Pharmacokinetics in Drug Discovery

In the following, the main tasks and activities of pharmacokinetics during the different phases of drug discovery are described, emphasising the exposure-driven approach of project support with integral PK/PD thinking and considerations. Other DMPK aspects are only touched upon and may be followed up in more detail elsewhere (Kerns and Di 2008; Tsaioun and Kates 2011; Zhang and Surapaneni 2012; Smith et al. 2012; Wang and Urban 2014). Although the chapter has been written with oncology projects in mind, the principles outlined below are also applicable to other indications and small molecule drugs.

2.1 Target Validation

For projects that are based on a novel, unprecedented disease hypothesis efforts to validate the new drug target start at the initiation of the project. For these so-called first-in-class projects, there are by definition no tool compounds available, and early evidence for the validity of the target also depends on the availability of suitable animal models which may either involve animals in which the target has been knocked out or significantly attenuated (si-RNA). In these projects, the confidence in the target accumulates throughout the entire drug discovery and development process with the ultimate evidence coming not until the clinical proof-of-concept (PoC) studies.

If the programme is going for a best-in-class approach, there is a high level of confidence in the target, and both tool compounds and relevant animal models are available. A rigorous interpretation of the role of the proposed target in the disease strongly benefits from the availability of exposure data of tool compounds in the animal studies. To assess the compound exposure, plasma samples are being collected from the animals, and, after sample preparation, the compound is quantitated by LCMS/MS analysis. Typically, a crude time course with just a few sampling time points covering the dosing interval suffices. Together with the in vitro data of the fraction unbound in plasma in the animal species, the unbound concentration–time profile in plasma is plotted against a relevant potency parameter, e.g. the unbound IC50 from in vitro tests to see whether the systemic levels reached in the animal study were in the range to cover the target and to elicit the desired effect (Fig. 2). Visualisations of this kind particularly help understanding (1) whether a negative experimental outcome was due to insufficient target exposure or an incomplete understanding of the target and (2) whether a positive outcome of the animal study is in line with the compound exposure at the proposed pharmacological target.

2.2 Lead Generation

Once there is a satisfactory level of evidence for a new drug target, a lead finding strategy is being pursued. This typically involves a high-throughput screen (HTS) of large compound libraries in order to identify hits/hit clusters that can serve as lead structures on which subsequently a full lead optimisation (LO) programme may be based on.

From a DMPK point of view, there are two main objectives in this phase: (1) support of medicinal chemistry in assisting hit cluster evaluation and ranking through identification of the DMPK liabilities and assessment of the optimisation potential of the compounds in each cluster and (2) support of in vivo pharmacology studies with exposure-based advice on dose and schedule design of PD and efficacy studies.

Once the hit-to-lead process has narrowed down the number of hit clusters to a few, about 3–5 compounds per cluster are being subjected to in vitro ADME assays, in particular metabolic stability in animal and human liver microsomes and/or hepatocytes, Caco-2 permeability and efflux as well as CYP inhibition using human liver microsomes and CYP induction potential based on the PXR assay (Roberts 2001, 2003; Tsaioun and Kates 2011; Zhang and Surapaneni 2012; Wang and Urban 2014). Suitable compounds which are also pharmacologically active are subsequently submitted to rodent PK studies in vivo to examine how the in vitro ADME liabilities translate to in vivo, to determine the PK parameters and to assess their behaviour in the whole organism (Li et al. 2013). This allows to elucidate what type of DMPK liabilities a hit cluster may carry, how many liabilities there are in a given cluster, whether all compounds tested show the same type of liabilities and whether there are structural variations which could serve as promising starting points for chemical optimisation. The power of this analysis increases with a clear differentiation between properties which are relevant for the whole cluster and properties which are seen in single compounds only. In addition, we have made the experience that HTS runs using cellular potency screens result in higher ADME quality hits compared to HTS based on biochemical assays. Although hits often seem more potent in the latter, hit clusters identified by cellular assays tend to be more drug-like right from the start, ultimately making it more likely to turn them into viable drug candidates.

Our experience has also shown that hit clusters carrying more than three independent compound liabilities are very difficult to optimise during the LO phase. Independent liabilities are, for instance, poor potency and selectivity, low aqueous solubility, strong efflux, high clearance, CYP inhibition as well as CYP induction. In contrast, low permeability, low metabolic stability and low oral bioavailability are not considered independent if the latter liability is the consequence of the former two, e.g. low bioavailability due to low intestinal absorption or high hepatic first-pass. Similarly, a short half-life may be the consequence of high clearance.

Programmes with a difficult chemical starting point generally tend to consume a substantial amount of effort in many functions involved without being able to

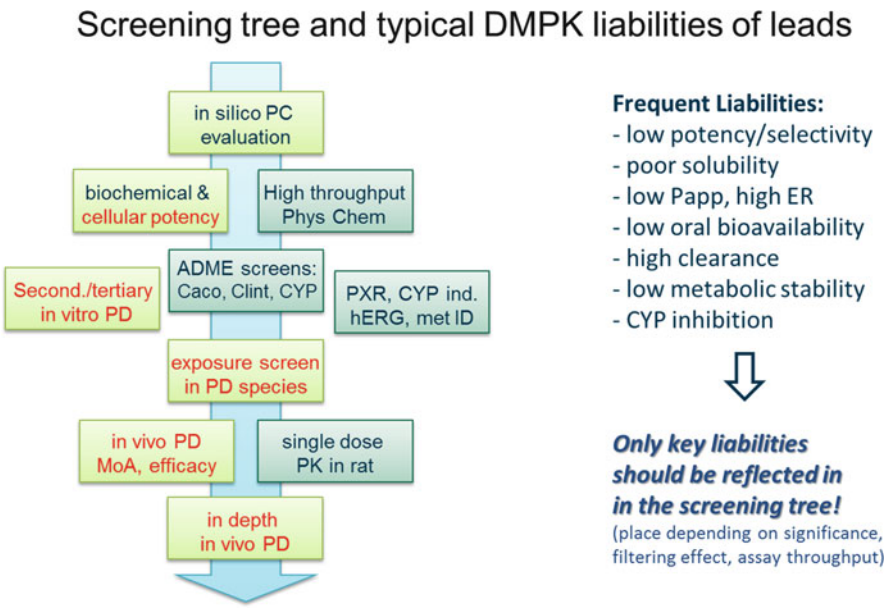

Fig. 4 Example of a screening tree for early lead optimisation. Typical DMPK liabilities which are most frequently seen in lead compounds are shown on the *right*. Tailored to the specific liabilities of the lead structures in each project, the most appropriate assays are selected and placed into the screening tree depending on the type and significance of the liabilities, and the filtering effect of the assay results to discard or progress compounds to the next tier of assays which also takes into account the complexity and throughput of the assays. The *green* and *blue colours* of the boxes reflect PD and DMPK assays, respectively. Highlighted in *red* are those assays and study types which provide input for the generation of PK/PD relationships between in vitro potency, unbound exposure, PD and/or efficacy readouts in animal studies

deliver in a viable drug candidate even if the team is given extra capacity and an extended period of time. In such instances, it is advisable to explore the chemical space by synthesising new compounds around the hit structure to assess the tractability of the chemical space in terms of how feasible it may become to improve the various liabilities in one chemical structure/molecule. Achieving only independent improvements of each liability in separate molecules may not be sufficient to forecast the effort needed during the LO phase and to increase the probability of success to find a viable development candidate.

The sound identification of the key liabilities of the lead cluster allows the design of a powerful and efficient screening tree for the LO phase. A typical example is shown in Fig. 4. The power of the screening tree often increases when focussing on the most relevant assays from the different functions and disciplines placed into a logic which follows the criticality of the assay with regard to the optimisation goal, the throughput of the assay and the filtering effect of the results on compound progression to the next tier of assays or studies.

Besides the identification of the liabilities of the hit cluster, the project team also defines the desired profile of the drug candidate as optimisation goal for the LO phase. The stepwise generation of a basic understanding of the relationship between the PK/exposure and the pharmacodynamics/efficacy is extremely beneficial already during this early phase of a programme. If the new programme is going to be a follow-up of an advanced preclinical or clinical compound, this is a must and forms the basis for the definition of the optimisation goals, together with the information on those liabilities or properties of the front-runner where the follow-up candidate has to improve on or differentiate against.

For a new programme with little or no precedence, early conductance of dedicated in vivo PK/PD studies have proven particularly informative to learn about the role of the target and the dynamics of target engagement for in vivo efficacy. This relationship can be studied already with compounds which are still suboptimal in terms of their PK properties, as long as sufficient exposure can be achieved in animals with unbound plasma concentrations approaching unbound in vitro IC50 values of target inhibition. For instance, highly potent compounds which still suffer from high metabolic instability/clearance and/or low absorption due to high efflux may be given at high doses in pharmacological in vivo studies, thereby overrunning/saturating these mechanisms. Sufficient plasma exposure can thus be achieved in animal PD models, allowing exploration of the relationship between unbound plasma concentrations, in vitro potency and in vivo efficacy readouts from quite early on. In case of very insoluble compounds, unusual formulations may be used as long as the excipients applied do not interfere with PD/efficacy readouts. The purpose of such studies is not to rescue pharmacokinetically insufficient compounds, but to extract as early as possible what type of in vivo concentration–time profile best enables the efficacy of a given target mode-of-action (MoA). Indeed compounds with a very short half-life are especially well suited to explore time dependencies in PK/PD relationships, e.g. to examine on/off requirements for target engagement.

The earlier and the better the understanding of the PK/PD relationship of the target is developing, the better the guidance that can be given to the project team. The impact may be severalfold: (1) Often there are diverse types of in vitro potency assays encompassing different biochemical and cellular systems with sometimes different assay conditions. Understanding the PK/PD relation helps to select the in vitro potency assay which is most relevant for in vivo efficacy. This should subsequently become the assay on which to base on structure–activity relationships during the LO phase. (2) Upon varying doses and administration schedules of PD/efficacy studies, different unbound plasma concentration–time profiles can be linked to differences in the in vivo coverage of in vitro potency/IC50 values. This may serve as first indirect evidence for target engagement and thereby will further strengthen the confidence in the target. (3) The PK/PD relationship allows delineating the type and shape of the plasma concentration–time profile needed to elicit intended pharmacodynamic effects. This information will in turn help to define the desired PK profile of the development candidate and to define what PK

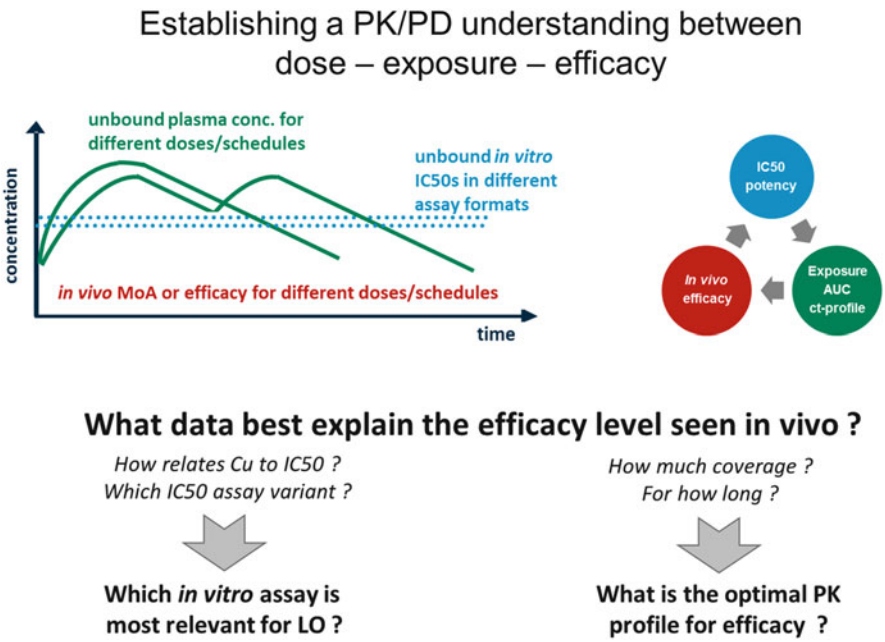

Fig. 5 Illustration of the iterative approach of applying different doses/schedules of a compound to explore the pharmacological activity in vivo based on defined MoA or efficacy readouts in relation to the coverage of the potency (e.g. unbound in vitro IC50) by the corresponding unbound plasma concentration–time profile. This information triggers a learning cycle to establish (1) what type of in vitro potency assay is most relevant for the in vivo activity and (2) what shape a concentration–time profile should have to enable the desired level of target engagement

properties of the lead compound(s) need(s) to be optimised in order to get there (Fig. 5).

2.3 Lead Optimisation

The decision to start the optimisation of a lead structure class endorses a significant investment into a project assigning the allocation of large amounts of resources in medicinal chemistry, pharmacology, drug metabolism and pharmacokinetics and many other disciplines to embark on a multidimensional optimisation of the chemical starting matter to improve the liabilities of the lead structure. The ultimate goal of the optimisation is to generate a drug candidate molecule which carries substantial evidence not only to be efficacious in a well-defined indication and patient population but also to be able to be administered safely and conveniently to humans.

DMPK efforts during the LO phase concentrate on those aspects which are critical (1) to change the PK profile of the compounds such as to enable efficacy, (2) to avoid/reduce the potential of the compounds to elicit safety risks and

drug–drug interactions (DDI) both as victim and perpetrator when given to patients with indication-specific co-medications and (3) to ultimately allow for a human efficacious dose and dosing schedule which can be formulated and administered in a way that is convenient for clinical use.

Based on the growing understanding of which elements in the concentration–time profile are driving efficacy (the so-called PK/PD driver) and the desired route and scheme of administration, it can be simulated which and how the PK parameters of the lead compound and its analogues need to be optimised in order to turn them into potential drug candidates (Fig. 5). In this phase, it is important to be able to answer key questions such as the following: Which pharmacological IC50 assay variant is most relevant and hence predictive for in vivo efficacy? How much and how long should the unbound plasma concentration stay above this in vitro IC50 to consistently see in vivo efficacy with minimal side-effects? The earlier and the better the team learns to answer these questions, the more they can concentrate their efforts on those parameters which really make a difference rather than getting lost in aspects which are not on the critical path and simply seem easy to be addressed at first sight. The more stringent a team is able to focus, the more rapid it will make progress.

Identification of the elements in the concentration–time profile that are driving efficacy (e.g. AUC, time over IC50, C_{max}, C_{trough}, concentration at a time) requires application of varying the doses and dosing schedules in animal efficacy studies that cover a dose range from full to medium and low/no efficacy. Split-dose studies (e.g. 50 mg/kg vs. 2×25mg/kg) are very useful as the AUC remains the same, but C_{max} and time over IC50 differ. If both schedules are equally efficacious, efficacy is more likely to be driven by AUC, while a higher efficacy of the latter may be suggestive of time over IC50 if this was longer in this schedule. This information can be used to guide PK optimisation depending on what aspect in the plasma concentration–time profile in the current compounds is still suboptimal. If the optimisation goal is to elevate unbound drug concentrations, ADME mechanisms to be optimised are aqueous solubility, intestinal permeability/efflux and/or metabolic clearance, whichever is/are the limiting factor(s) for a given class of compounds (Wang and Urban 2014; Reichel 2014, 2015). Noteworthy, the fraction unbound in plasma or tissues is not an optimisation parameter (Reichel 2009; Smith et al. 2010). If the optimisation goal is to increase the half-life of the LO compounds in order to extent the time over IC50 achievable by a single dose, the parameters to look out for improvements are clearance and volume of distribution. Clearance can be reduced by increasing the metabolic stability of the compounds or reducing the renal or biliary elimination (whichever is the main clearance pathway). The volume of distribution may be increased by making the molecules more lipophilic or basic (Smith et al. 2012).

Focussing on the critical path from a PK point of view means to concentrate on those aspects which really impact the concentration–time course of the LO compounds and their technical DMPK profile in the desired direction. This involves the generation of structure–activity relationships (SAR) and structure–potency relationships (SPR) to explore the chemical space and to elucidate those structural

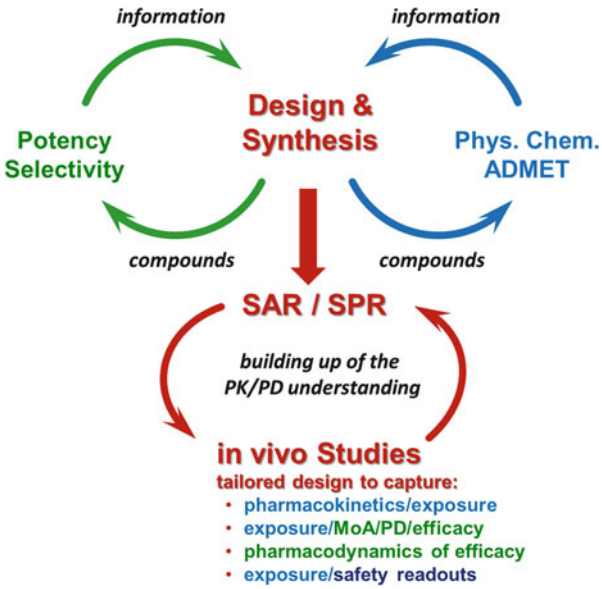

Fig. 6 Illustration of the design–make–test–analyse cycle in the LO phase. The in vitro assay in pharmacology and DMPK have to run at the same throughput and turnaround times to be able to synchronise the generation of structure–activity (SAR) and structure–property relationships (SPR) to avoid bias in the chemical synthesis. Optimised compounds are then submitted to in vivo studies (1) to examine the overall PK behaviour of the compound and (2) to evaluate the pharmacological activity in vivo in relation to unbound plasma concentrations and the in vitro potency (see Fig. 5). If possible safety readouts can be included in the in vivo studies. Depending on the level of understanding of the pharmacology of the target and the animal disease model, mode-of-action studies which can be performed after single doses or more chronic efficacy studies with repeated dosing schedules are carried out

moieties which carry the highest potential for potency and selectivity (via SAR) and optimal physicochemical, DMPK and safety properties (via SPR). It is beyond the scope of this chapter to discuss the standard ADME and safety in vitro assays which have been described extensively in the literature (Kerns and Di 2008; Meanwell 2011; Tsaioun and Kates 2011; Zhang and Surapaneni 2012; Smith et al. 2012; Wang and Urban 2014). It is important that these assays run at high throughput and with short turnaround times which are fully in line with design–make–test–analyse learning cycles (Plowright et al. 2012) that are jointly driven by medicinal chemistry, pharmacology and DMPK (Fig. 6).

A small core team with one representative from each of the three functions has been recognised to be the most efficient set-up in this process, in particular if they work along a clear hypothesis-driven way: i.e. defining a decision-related question for each compound prior to synthesising and subsequently submitting it to the relevant screening assays so to be able to rationalise their thoughts and to gradually transform the rapidly expanding amount of information into working knowledge rather than simply filling data bases very efficiently.

Once promising compounds have emerged from the early optimisation cycles, animal PK studies will be performed to verify the improvements made in vitro in the whole animal in vivo. Establishing and verifying the link between in vitro and in vivo (IVIVC) is important to ensure that the screening assays have the desired impact and that the screening tree is still relevant. Otherwise, the screening tree needs readjustment to respond to the chemical space and issues of the current compounds and to return on the critical path. While there are simple and convenient tools to examine IVIVCs such as correlation analysis between two or a few more properties, more sophisticated tools such physiologically based PK (PBPK) models offer the advantage to propagate in vitro ADME data through an in silico representation of the whole organism which allows simulation of the impact a given property change may have on the concentration–time profile (Parrott et al. 2005; Lüpfert and Reichel 2005; Peters et al. 2009; Chen et al. 2012; Rostami-Hodjegan 2012; Jones et al. 2015). The purpose of the application of PBPK modelling in this phase is not to be predictive of human but to diagnose ADME liabilities that are key to be improved and subsequently to aid the identification of compounds with an optimal balance of properties (and an acceptable compromise of their insufficiencies) to enable the desired concentration–time and PK profile.

Compounds which are expected to provide sufficient target exposure in animal PD models qualify for efficacy studies to explore their pharmacological profile in vivo. Prior information from the in vivo PK and/or pilot exposure studies supports the design of these studies in terms of suggesting doses and schedules which are most likely to be efficacious and for suggesting suitable time points for sampling of plasma and/or tissue. Exposure measurements which allow to capture a time course throughout the dosing interval are important and allow to dynamically link the concentration–time profile with the effects seen (or not seen). Dedicated dose–response studies with different dosing schedules are particularly powerful for PK/PD modelling and simulation (Gabrielsson et al. 2009, 2010, 2011; Bueters et al. 2013; Tuntland et al. 2014). If the design of these studies has been carefully thought through, important pieces of information can be extracted giving answers to questions such as the following: Can efficacy be linked to the unbound plasma concentrations? What element(s) in the plasma concentration–time profile drives efficacy (i.e. the PK/PD driver)? For how long should the target be exposed/engaged to elicit a certain level of efficacy? What would be the unbound concentration–time profile we like to see in humans to be efficacious in the clinic?

Single-dose studies with MoA readouts in target tissue can be very informative of target exposure, target engagement and subsequent expression of pharmacology when pharmacological readouts are taken dynamically, i.e. followed along a time course after dosing together with plasma and tissue samples to be analysed by means of LCMS/MS for the corresponding compound concentrations. The relation between the compound concentrations in plasma and target tissue and the extent and duration of target inhibition needed for efficacy forms the basis for quantitative PK/PD models which ultimately will be used to estimate the human efficacious dose.

While it is important to keep a clear focus on the critical path and to develop a growing understanding of the relationship between pharmacokinetics/exposure and pharmacodynamics/efficacy, there are a number of pitfalls on the way to identify a viable drug candidate:

1. The potency trap. Care should be taken that the screening tree is not steering chemical synthesis into the potency trap. Highly potent compounds often carry too much lipophilicity resulting in a prohibitive ADME profile which in most cases cannot be improved without losing potency altogether. Using potency measures such as the ligand binding efficiency index, where potency gains merely by lipophilicity increase are punished, can be very useful indicators to avoid this pitfall (Reynolds et al. 2008; Hopkins et al. 2014). Also, putting due emphasis on cellular potency readouts with demonstrated relevance for in vivo efficacy rather than on potency values from biochemical assays with recombinant target proteins helps avoiding this trap. Instead, biochemical assays should be used primarily for improving the selectivity of the compounds.
2. The fast and easy way. Beyond the many easy to synthesise chemical modifications, try also to get hold of more challenging to synthesise molecules in the chemical series if they are indicated by SAR/SPR analyses. If small structural changes only bring about small improvements, larger chemical modifications might be more fruitful. They also allow exploration of the distant corners in the chemical space and may direct teams on the (unexpected) path to the ultimate development candidate (Lücking et al. 2013; Hartung et al. 2013).
3. Getting lost in too much. Attempting optimisation of all liabilities at once often proves to be overtly challenging, not so much in terms of capacity but in terms of rationalising too many SAR and SPR relationships simultaneously. It may be more satisfactory to focus on the resolution of the most crucial issues initially and address other issues once significant improvements have been achieved. The then new chemical matter might no longer suffer from other liabilities or may have other issues to take care of. The paradigm of "all compounds in all assays as early as possible" produces a lot of data and noise, and the overwhelming amount makes it difficult to extract useful knowledge for decision-making. Well-defined rational design–make–test–analyse cycles with a relevant question behind each compound and every assay the compound is submitted to turn out to be more instructive besides saving capacity (Ballard et al. 2012; Plowright et al. 2012).
4. Losing sight of the relevant issues. The validity of the screening assays in terms of delivering real improvements on the key liabilities should be repeatedly checked by in vivo studies during lead optimisation. For instance, IVIVC between solubility and/or Caco-2 permeability/efflux and oral bioavailability, and metabolic stability and total clearance should be confirmed throughout the LO phase to make sure that a significant improvement seen in vitro indeed brings about the anticipated improvement in vivo. This ensures that the screening tree is still on the right track and indicates if new issues have turned up in the current compounds which need to be taken care of in a different set of screening assays.

5. Clinging on for too long. Sometimes even excessive optimisation efforts in medicinal chemistry, pharmacology and DMPK do not result in sufficient improvements of the current compounds compared to the lead structures. Better results on one liability lead to a deterioration of one or more of the other liabilities and vice versa. One issue may be solved, but other issues surface up and no way out seems on the horizon where at least the most important liabilities can be resolved in one molecule. When the team has arrived at such a situation, it may be hard but unavoidable to ask the "killer question" (Cook et al. 2014) rather than continuing and hoping for the "lucky shot". A stringent exploration of the chemical space at the virtual interface between ADME space and potency/selectivity space helps supporting at a clear Go/Nogo decision. Abandoning an un-optimisable lead structure in due course allows redirecting of valuable LO resources to other programmes where they will have more impact. Projects should not be terminated, however, without extracting lessons learned about the reasons for failure, being related either to the target or to the chemical matter, each having important but different implications for future programmes. Convincing evidence for the former reason is the demonstration of target exposure and expression of pharmacological activity (MoA) with consistently no impact on the efficacy in animal models.
6. Stay open minded. While the virtue of a rational, hypothesis-driven way of working with continuous integration of data and extraction of knowledge and understanding is very obvious, there are also situations where the data do not seem to make sense or do not seem to fit in the current thinking. Trying to understand such discrepancies instead of ignoring them is important and can be very rewarding. They might either be due to experimental issues which need resolving to make the assay more relevant or they may point to a missing piece in the understanding of the project team which can turn out to become crucial for the progression of the project. Face-to-face brainstorming discussions in small groups tend to be more productive than trying to resolve such issues by electronic communication means. Not only may interactive discussions be more productive, they are also more likely to bring out creative ideas and solutions and even may be starting points for novel compounds, novel targets and indeed new projects.

After several LO cycles, the current compounds will carry significant improvements on the liabilities seen in the original lead structures. Arrival at this stage starts the late LO phase which besides continued screening efforts embarks on a broader examination of the most promising compounds in vivo, e.g. more sophisticated animal PD and efficacy models, non-rodent PK studies as well as pilot toxicology and safety pharmacology studies. These activities gradually lead into the phase of candidate selection and profiling.

2.4 Candidate Selection and Profiling

While the LO phase embarks on changing particular properties in a molecule, the candidate selection phase concentrates on a broad characterisation of the compounds that are finally emerging from the optimisation efforts of the programme. The purpose of the characterisation is to examine whether one of the most promising molecules qualifies as potential drug candidate. Apart from an in-depth characterisation by pharmacology, pharmacokinetics and drug metabolism, the compounds are also scrutinised by toxicology, safety pharmacology and formulation development (see, for instance, Pelkonen et al. 2002, Zhang and Surapaneni 2012, and Wang and Urban 2014). In the following, attention is paid to those PK aspects which relate to efficacy and safety: (1) predicting the PK in human, (2) estimating the human therapeutic exposure, (3) predicting the therapeutic dose in patients and (4) estimating the therapeutic window in human. This phase will also identify the potential DMPK-related risks which have to be addressed specifically during preclinical and clinical development to de-risk the programme and to allow assessing of the future investments needed and the likely probability of success.

2.4.1 Prediction of Human PK

The anticipation of the PK behaviour in humans has the intention to predict the exposure and plasma concentration–time profile in humans for a given dose applied to patients via the intended route of application. This prediction is fundamental to estimate the human therapeutic dose as well as the expected therapeutic window.

The prediction of the PK in humans is a complex exercise and cannot be dealt with in detail in this chapter. It involves (1) the generation of in vitro ADME data both in animal and in human systems, in particular with regard to clearance mechanisms; (2) in vivo PK studies in at least 2–3 species, including rodent and non-rodents; and (3) the integration of this data into a framework which allows to predict the PK profile in humans (Beaumont and Smith 2009; van den Bergh et al. 2011; Grime et al. 2013). The methodologies and approaches have been reviewed and evaluated extensively across industry in a recent PHRMA initiative (Poulin et al. 2011). We have made good experiences with a combination of allometric interspecies scaling and human in vitro data including simultaneous fitting of the plasma concentration–time profiles of the animal species and physiologically based approaches. Human PK predictions are carried out for the following PK parameters: clearance (CL), volume of distribution (V_{ss}), area under the plasma concentration–time curve (AUC), elimination half-life ($t_{1/2}$), oral bioavailability (F%) and the highest and the lowest plasma concentration (C_{max} and C_{min} or C_{trough}, respectively). In addition, plasma concentration–time profiles will be simulated for the intended route of application.

While allometry generally performs well for volume of distribution when corrected for plasma protein binding, this approach performs clearance less well, particularly if there are species differences in the rate and/or mechanism(s) of clearance which cannot be corrected for by plasma protein binding, intrinsic

metabolic clearance or other correction factors (Mahmood 2005; Beaumont and Smith 2009). In such cases, single species scaling can be applied to derive different scenarios of the predicted human PK parameters and plasma concentration–time profile. Confidence in the predictions depends as much on experience, context information and tacit knowledge from this and other programmes as it does on actionable study suggestions which allow to add further confidence when going ahead towards or during preclinical development.

Another very important aspect, although only indirectly related to human PK prediction as such, should be mentioned here. Up to this point in time, the oral bioavailability generally has been examined from oral solutions based on rather undefined material. In order to estimate the oral bioavailability from well-defined compound material (e.g. microcrystalline material of defined particle size), the relative oral bioavailability between the two states is being determined in rats at an equivalent of the human therapeutic dose. A relative oral bioavailability of below 50% indicates the need for special formulation efforts to overcome potential dissolution limitations of the drug material, while high values indicate that a standard immediate release tablet formulation is feasible to achieve the anticipated exposure in humans (Muenster et al. 2011).

2.4.2 Estimation of Therapeutic Exposure in the Patient

Although tempting, the human therapeutic exposure cannot simply be taken from the efficacious exposure in an efficacy study, even if corrections are made for plasma protein binding to obtain the unbound AUC and plasma concentration–time profile and for possible species differences in potency.

In contrast, the estimation of the pharmacologically active concentration–time profile in humans should be based on a set of dedicated PK/PD studies with quantitative data analysis and evaluation. These should include studies (1) to identify or confirm the PK/PD driver of efficacy of the development candidate in relevant PD and/or efficacy models and (2) to quantitatively describe the relationship between the plasma concentration–time profile and the level of efficacy seen for different doses and dosing schedules in models which are relevant for the intended patient population. The predictive power and hence the confidence in PK/PD model increases by incorporating the PK/PD understanding which has evolved in the LO phase not only on the candidate compound but also on other compounds in the project. If available, front-runner compounds or competitor drugs can be very valuable to develop and validate prediction algorithms. In oncology, for example, the translatability of the efficacious exposure in tumour xenograft studies in mice to the efficacious exposure in human patients has been demonstrated using a set of antitumour drugs used in human patients (Simeoni et al. 2013). This approach quantitatively links the ability of different drugs to inhibit tumour growth to the exposure in a set of dose–response studies based on an in vivo potency measure which directly correlates with the human efficacious AUC of the antitumour drugs tested (Rocchetti et al. 2007). Capturing the tumour growth rates following different dosing regimens also allows the estimation of experiment-independent parameters, thereby increasing the confidence in the model parameters. PK/PD

models to estimate the efficacious exposure can be further enhanced by integration of target engagement markers or subsequent MoA readouts and by attempting to establish a temporal link between the expression of pharmacological activity upon target binding with more "distant" efficacy endpoints such as tumour stasis or regression (Yamazaki 2013; Yamazaki et al. 2015; Venkatakrishnan et al. 2015).

Besides the confidence in the relevance and translatability of the results from animal models to the human situation, the relevance of unbound plasma concentrations for the concentrations in the effect compartment needs to be understood. For targets residing in the body's periphery with unrestricted proximity to the blood circulation, the time course of the unbound plasma concentrations most likely will correspond to the unbound concentration at the target site. This can be demonstrated experimentally by plotting the time course of total plasma vs. total target tissue concentrations. If they run in parallel, the unbound concentrations will be the same in both compartments, i.e. the unbound plasma concentration fully represents the free concentration of the compound at the target site. This information on plasma-target site equilibrium is very important for clinical studies where generally only plasma concentrations are accessible. If the target site in the body is not in direct correspondence with the plasma compartment, a possible exposure difference and/or time delay should be evaluated (Gabrielsson et al. 2009, 2011). For targets which are not in proximity to the general blood circulation or even locate behind a physiological barrier, e.g. CNS, additional data are needed to translate unbound plasma concentrations into relevant effect compartment concentrations (Reichel 2009, 2014, 2015).

PK/PD modelling based on popPK approaches combining different, independent experiments and using the data points from individual animals is very powerful to reduce noise and to extract experiment-independent parameters for the predictions.

The predicted therapeutic exposure in humans has also to be consistent with the existing knowledge of the target biology. Predictions carry particularly high confidence if they are based on experimental evidence for target engagement and expression of pharmacological activity (MoA) in an animal model which is relevant for the intended indication and patient population.

2.4.3 Prediction of Human Therapeutic Dose

Conceptually, human dose estimation is simple (Fig. 7): The predicted PK, i.e. dose–exposure relationship in humans from above, is used to estimate a dose/dosing schedule for humans which is anticipated to mimic the unbound concentration–time profile that is expected to be efficacious as well as safe in humans (Heimbach et al. 2009; Venkatakrishnan et al. 2015).

The accuracy of the predicted dose depends as much on the prediction of the human PK as it does on the estimated therapeutic exposure. The estimation of the human therapeutic dose often results in a range originating from different scenarios that reflect the most relevant uncertainties in the current understanding and set of data available. These may relate to both the prediction of the human PK and the estimation of the human therapeutic exposure. The use of PD or disease models in different preclinical species helps in eliminating any species differences between

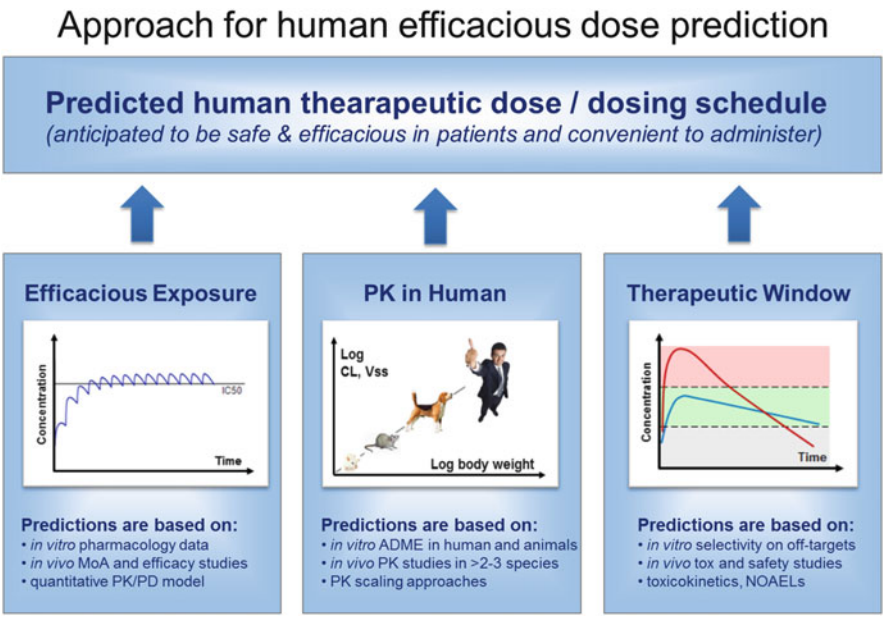

Fig. 7 Schematic illustration of the prediction of the anticipated efficacious therapeutic dose in human. The approach integrates information on (1) the therapeutic exposure that is expected to be pharmacologically active in patients (*left panel*), (2) the predicted PK parameters and plasma concentration–time profile in humans (*middle panel*) and (3) the estimated therapeutic window based on the no adverse effect levels (NOAELs) in animal toxicology and safety pharmacology studies (*right panel*)

pharmacokinetics and pharmacodynamics, thereby refining the extrapolation to the human situation. For best-in-class projects, the confidence in the dose estimations may be further increased by applying the same prediction algorithm to competitor compounds with proven clinical efficacy and using possible differences as correction factors (Lowe et al. 2007).

The design of PK/PD studies which specifically elude on possibilities for intermittent dosing schedules along with information on the mode-of-action in target cells and/or in surrogate cells (e.g. in the circulation) as well as relevant PD readouts will be informative for alternative scheduling options. In vitro washout experiments where the time course of the cellular activity is followed after removal of the compound may provide important input to model such alternative intermittent dosing regimens with high probability for efficacy. Such studies are also helpful to delineate any temporal disconnects between pharmacokinetic and pharmacodynamics as a consequence of time events along the downstream pharmacological cascade. In oncology, intermittent dosing schedules are particularly attractive as they may increase the therapeutic window of the antitumour agents.

Predictions on alternative treatment schedules may also support the strategic positioning of the drug for clinical use and/or to support treatment schedule or

dosing related competitive advantages. The prediction of the therapeutic dose when the drug candidate is going to be used in combination with other drugs is an important consideration for the predictions in oncology (Simeoni et al. 2013). For drug combinations, it is important to examine whether the interaction is additive or synergistic (Schmieder et al. 2013) and to exclude that the additional benefit is due to pharmacokinetic drug–drug interactions, e.g. via CYP inhibition. For compounds where pharmacologically active metabolites are expected to significantly contribute to efficacy, these need to be incorporated quantitatively into the PK/PD models used for the prediction of the human therapeutic dose of the proposed drug candidate.

2.4.4 Estimation of the Therapeutic Window in Human

The therapeutic window is the ratio between the unbound exposure which does not yet show first signs of toxicity and the unbound exposure that is needed for efficacy. A first estimate of the therapeutic window derives from the comparison of the unbound efficacious AUC (from animal PD/efficacy studies) with the unbound AUC in toxicology studies/species which did not elicit adverse effects, i.e. NOAELs. In addition, a range of safety pharmacology studies is performed to estimate plasma concentrations which affect key physiological functions. As conservative estimates, the maximal unbound plasma concentrations are used to evaluate safety risks, i.e. C_{max},u, which are in turn compared to unbound C_{max} concentrations predicted for the highest efficacious dose in humans.

Whereas regulatory safety pharmacology and toxicology studies are being performed prior to entry into human according to authorities' guidelines, potential development candidates are evaluated in non-GLP pilot toxicology studies. They encompass, for instance, 2-week toxicology studies with three dose levels in rodents starting from the dose corresponding to the efficacious unbound AUC with the other two doses representing different multiples thereof. The study design is project and compound specific, depending on prior knowledge from in vivo PK and PD studies, the indication space and the intended patient population.

The selection of the species for the toxicological examination of the compound depends on the metabolic characterisation of the compound, i.e. species similarities in the metabolite pattern with that generated by human hepatocytes. If a human-specific metabolite is not generated in the tox species, it may be synthesised by medicinal chemistry and applied directly in additional study arms. Also important is the achievability of multiples of exposures using microcrystalline material and formulations which can be used later by regulatory toxicology studies. In cases where this poses difficulties, special activities from formulation development are required to progress the project.

The estimation of the therapeutic window also affects the evaluation of potential drug–drug interaction (DDI) risks, depending on whether potential AUC increases would stay within a large therapeutic window or go beyond, thereby putting patients on co-medications at risk. DDI risks may arise from CYP inhibition and/or induction (Prueksaritanont et al. 2013) but also from interactions with

transporter proteins at the level of absorption, distribution or elimination (Giacomini et al. 2010).

While current estimations of the therapeutic window are often static, i.e. based on AUC or C_{max} values, dynamic modelling approaches are used more often as they better reflect the dynamics of the concentration effect relations both for efficacy and safety readouts (Muller and Milton 2012; Parkinson et al. 2013).

2.4.5 Identification DMPK-Related Development Risks

Experience shows that there is hardly any project without issues. The better and the earlier these issues – or risks – are known, the better one can react on them through dedicated de-risking measures. DMPK-related development risks may relate to a number of aspects such as:

1. Safe use in humans, e.g.:
   - Potential for drug–drug interactions as perpetrator and/or as victim, occurrence of toxic or reactive metabolites
   - Nonlinear PK in the therapeutic dose range
   - Potential for large interindividual variability due to involvement of polymorphic drug-metabolising enzymes
   - Drug accumulation due to very long half-life
   - Clearance pathway not compatible with special patient populations, e.g. renal insufficiency

2. Efficacious use in humans, e.g.:
   - Intestinal absorption issues
   - Formulation-related issues
   - CYP induction/autoinduction
   - Potential for large interindividual variability due to involvement of polymorphic drug-metabolising enzymes

3. Difficulty to achieve multiples of exposure of the parent drug and relevant human metabolites in the species used for in vivo toxicology and safety assessment, thereby limiting the exposure range in regulatory toxicology studies and hence complicating or even restraining dose escalations during clinical development

In addition to many other aspects which are not the subject of this chapter, the decision to start preclinical development takes into account the complete ADME and DMPK characterisation of the compound, the anticipated PK behaviour of the compound in humans, the expected therapeutic exposure and predicted dose regimen in patients, the estimated safety margins and the development risks identified. Any risk that has been identified is a strength rather than a weakness of the project as only the issues which have been identified yet allow proposals of appropriate risk mitigation steps at the various phases of preclinical and clinical development.

A problem-solving attitude to design and subsequently test project-specific in vivo study protocols integrating PK, PD, BM, Tox endpoints can provide extremely useful information for the understanding of and confidence in the compound and its subsequent progression into drug development. Working in cross-functional teams with preclinical and clinical experts allows tailoring of a clear path forward to resolve any issues identified and to carve out an early clinical development plan that permits an explicit testing strategy of the disease hypothesis in POC study designs that are powerful and conclusive and unbiased by exposure concerns.

It has to be noted that the handover of a project from discovery to development is not a point transition. Even though the responsibility during development is taken on by development functions, discovery PK continues to accompany the progression of the project and to contribute with their knowledge and expertise to resolving any issues that are coming up. Once human PK data from phase I studies are available, these are used by discovery PK to compare them with the predictions made and to explore any deviations observed. These comparisons are very rewarding and an important source to sharpen the tools and approaches to predict the PK behaviour in humans. Similarly, human data related to pharmacodynamic or efficacy readouts will be used to revisit the PK/PD relationships made in order to derive lessons learned and to grow confidence in the approach for future programmes.

3 Summary and Outlook

Since its foundation, discovery pharmacokinetics has moved a long way from starting off as service function which served to improve basic PK parameters such as clearance, half-life and bioavailability in potency-optimised compounds in response to the excessively high attrition of 40% due to poor pharmacokinetic properties of drug candidates during clinical testing in the 1980s and early 1990s (Kennedy 1997). Even in its rather service-oriented role, the impact of addressing ADME issues from early on in drug discovery programmes was astonishing, reducing PK-related failure rates down to 10% in about a decade (Kola and Landis 2004). The impressive effect has owned discovery DMPK not only full acceptance; it is now seen at the core of drug discovery being an integral part of every drug discovery project (Smith 2011).

Still, beyond designing of adequate DMPK properties into the new drug candidates, there is a great deal of contribution left to help reducing the unacceptably high attrition due to insufficient efficacy and unacceptable safety (Kola and Landis 2004; Morgan et al. 2012; Cook et al. 2014). A rigorous application of pharmacokinetic principles in order to gain a quantitative understanding of the role of new drug targets in proposed disease mechanisms and subsequently in the patients by means of rigorous PK/exposure and PD/efficacy modelling and simulation approaches will be very powerful to increase the success rate of drug discovery and development. Optimising ADMET properties of lead compounds during the

LO phase in conjunction with building up of a growing understanding of those PK/PD properties that are required to elicit efficacy should be a key guiding principle during the LO phase. The generation of quantitative PK/PD models is a powerful approach to support the selection of adequate candidate drugs for preclinical and clinical development. The availability of such models will facilitate understanding of the relationship between target exposure and target engagement, the subsequent expression of pharmacological activity along with the proposed mode-of-action as well as pharmacodynamic and efficacy endpoints first in animal models and ultimately in patients (Danhof et al. 2007, 2008). Supporting drug discovery and development by exposure-based modelling and simulation (M&S) activities along the various phases of the project has been proposed to be a very powerful means to increase the success of clinical candidates (Morgan et al. 2012; Visser et al. 2013; Cook et al. 2014; Jones et al. 2015).

In oncology, for example, empirical and mechanistic PK/PD modelling and simulations of new antitumour agents has been demonstrated to add significant value to (1) the understanding of the target biology, (2) the confidence in a new disease hypothesis, (3) the identification of optimal doses and dosing schedules and (4) the selection of responsive patient groups (Venkatakrishnan et al. 2015). Translational modelling of preclinical tumour growth inhibition data from animal models to the effects in patients with regard to surrogate response markers, tumour growth inhibition and progression-free survival is an area which can benefit strongly from back translation of incoming clinical data to the discovery teams. Currently, M&S activities which aim at establishing a quantitative link between PK/exposure and mechanistic or semi-mechanistic models of biological pathway modulation are rapidly growing (Gabrielsson and Hjorth 2012; Yamazaki 2013; Venkatakrishnan et al. 2015).

Ultimately, such quantitative pharmacology models may not only be predictive of the effect of the inhibition of one target; they may also allow to simulate the interference of a drug or a combination of drugs with a network of targets, thereby giving rise to the discovery and development of new generations of medicines for treating diseases beyond the classical one drug–one target–one disease paradigm.

Although, today, we are still a long way from understanding and translating cellular effects to pharmacodynamic and safety-related effects in tissues and further to clinical readouts, the exposure-based PK support is a powerful approach that helps advancing (1) the discovery, optimisation, selection and characterisation of high-quality drug candidates with PK/PD properties that carry sufficient potential to show efficacy and safety in patients at the predicted dose and dosing scheduling and (2) the development through ultimately allowing a more rigorous testing of new disease hypotheses in patients without bias due to uncertainty in target exposure, making clinical proof-of-concept studies much more powerful than in the past.

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Nonclinical Safety and Toxicology

Claudia Stark and Thomas Steger-Hartmann

Contents

Abstract

Nonclinical safety pharmacology and toxicology testing of drug candidates assess the potential adverse effects caused by the drug in relation to its intended use in humans. Hazards related to a drug have to be identified and the potential risks at the intended exposure have to be evaluated in comparison to the potential benefit of the drug. Preclinical safety is thus an integral part of drug discovery

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and drug development. It still causes significant attrition during drug development. Therefore, there is a need for smart selection of drug candidates in drug discovery including screening of important safety endpoints. In the recent years, there was significant progress in computational and in vitro technology allowing in silico assessment as well as high-throughput screening of some endpoints at very early stages of discovery. Despite all this progress, in vivo evaluation of drug candidates is still an important part to safety testing. The chapter provides an overview on the most important areas of nonclinical safety screening during drug discovery of small molecules.

Keywords
Discovery toxicology · Drug discovery · Lead optimization · Nonclinical · Safety pharmacology · Safety screen

1 Introduction

Nonclinical safety and toxicology testing of drug candidates target on assessing the potential adverse effects caused by the drug in relation to its intended use in humans. Hazards related to the compound have to be identified and the potential risks at the intended exposure have to be evaluated in comparison to the potential benefit of the drug. In order to secure a thorough assessment of the nonclinical safety of the drug, multiple aspects have to be covered during the development of a drug.

This includes safety pharmacology, which addresses effects on organ function driven by primary or secondary pharmacology. Mandatory organs to be tested are the central nervous system, the cardiovascular system, and the respiratory system – the so-called core battery. In addition, supplemental testing may be performed addressing other organs, where a specific concern exists toward an impact of the drug on the organ function. Safety pharmacology studies are often performed as stand-alone studies, but they may also be integrated in general toxicity studies, which are best established for anticancer drugs (Authier et al. 2013).

Toxicology testing includes general toxicity after single or repeated administration of the drug including histopathology, the genotoxic and carcinogenic potential of the drug, and the impact on male and female fertility as well as embryo-fetal and postnatal development. In addition, potential local effects of the drug at the administration site, as well as specific aspects like phototoxicity, abuse potential, and immunotoxicity, have to be tested in case of concern. Timing of the safety studies in relation to clinical trials is largely harmonized and concisely described in ICH M3 (R2) (2009). In addition, the occupational safety during production and the environmental safety of the drug have to be assessed.

For the nonclinical safety testing of pharmaceuticals as prerequisite for the start of clinical trials and for marketing application – an area also referred to as regulatory testing – large sets of guidelines exist, which describe testing procedures

as well as recommendations for the extent of testing. The OECD guidelines on testing methods[1] give recommendations on experimental details such as minimum animal numbers and parameters to be tested as well as reporting of the results, which can also be applied for drug testing. In addition, there are specific guidelines for pharmaceuticals, most important being the ICH guidelines[2] addressing safety aspects, quality aspects (e.g., assessment of impurities), as well as multidisciplinary aspects. In addition, there are regional guidelines, e.g., those published by EMA[3] and FDA[4], also covering aspects not addressed in ICH guidelines. The regulatory safety testing has generally to be performed according to good laboratory practice (GLP), which includes testing in a test facility certified for the specific GLP studies and using test material according to GLP standards.[5]

For the nonclinical safety assessment in the process of drug screening and drug candidate selection, there are no formal regulatory requirements, neither with regard to the type of testing to be performed nor with regard to the quality of such data. As a consequence, the screening program is generally designed to support the choice of the best candidate by early identification of important development hurdles. There is a lot of space for tailor-made approaches based on the target, the chemical class of compounds, the intended therapeutic indication, and also the previous experience within these mentioned fields. The extent of screening largely depends on the need for differentiation between candidates as well as the need for early de-risking of specific liabilities. There are some areas, which are responsible for a high attrition rate and for which methods are available allowing for high throughput. Thus these endpoints are often recommended to be included into an early screening cascade prior to start of lead optimization. Examples for such endpoints are cytotoxicity (EC_{50} of cytotoxicity on nontarget cells vs. EC_{50} of primary pharmacodynamics effect on target cells), mutagenicity, and hERG current inhibition or hERG binding. For follow-up candidates, additional toxicity screening may be included depending on the nature of the findings with previous front-runner compounds. Examples for such additional screening are phospholipidosis and mitochondrial toxicity (Kramer et al. 2007). At the stage of candidate selection, in vivo pilot toxicity studies are still state of the art to derive the most predictive data for later preclinical and clinical development.

As mentioned above, the preclinical development program for the preparation of the initial clinical trials (FiM: first in man) is set forth in regulatory guidelines. The focus in the following sections will therefore be an overview of most important

[1] http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects_20745788

[2] http://www.ich.org/home.html

[3] http://www.ema.europa.eu/ema/index.jsp?curl=pages/regulation/general/general_content_000397.jsp&mid=WC0b01ac058002956f

[4] http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/default.htm

[5] http://www.oecd.org/chemicalsafety/testing/oecdseriesonprinciplesofgoodlaboratorypracticeglpandcompliancemonitoring.htm

areas for nonclinical safety *screening* of small-molecule drug candidates during discovery phase before start of nonclinical regulatory GLP studies. For large molecules ("biologics"), other screening strategies may be applied (e.g., immunogenicity screening, human tissue cross-reactivity studies). Toxicity of biologics is mostly related to the mode of action with classical off-target effects not frequently observed. The basic requirements for nonclinical safety assessment are described in ICH S6 (R1) (2011). However, these compounds are beyond the scope of this overview.

2 Molecular Off-Target Interaction

Screening for off-target interaction at a relatively early stage of the drug discovery process is important. The binding affinity and interaction of the candidate with structures closely related to the intended target (e.g., other receptors or enzymes of the same family) are determined as part of the primary pharmacodynamics characterization in order to define the specificity of a targeted pharmacological approach. An important aspect of secondary pharmacodynamics is the understanding of the expression profile of the pharmacological target in humans and nonclinical models used in safety evaluation. This can be done by literature mining; biochemical, cellular, genomic, and proteomic profiling; and systems biology approaches (MacDonald et al. 2006; Prokop and Michelson 2012). These areas of drug evaluation are evolving, but there are still significant gaps in knowledge and technologies.

In addition, evaluation of the interaction with other important protein targets is needed as part of pharmacological characterization. Off-target effects may result from interaction of the drug candidate with receptors, ion channels, transporters, enzymes, or other protein targets, which may cause significant adverse effects. Although the interaction of a drug candidate with the target protein at the intended binding site can be modeled and the 3D structure of the drug candidate is mostly available, the prediction of possible off-target binding based on a proteome-wide drug-target network using in silico tools is not yet routinely done because of the complexity of the analysis (Xie et al. 2011). Today, in vitro screening is still the method of choice by testing binding to and interaction with a broad panel of important human receptors, transporters, ion channels, and enzymes (Denton et al. 2012). Standard panels for this type of test are offered by various vendors. As a first step, a simple binding assay is generally sufficient. When binding is observed at relevant concentrations (IC_{50}-value), the potential functional impact on the target structure can be assessed – inhibitory or antagonistic activity vs. activating or agonistic activity. Such data may support compound selection in various phases of drug discovery; can also guide decisions on the need for additional nonclinical safety testing, for example, by expanding the safety pharmacology testing panel to check the in vivo relevance of such findings (see ICH S7A 2000); and can also be helpful in mechanistic explanation of possible effects found in subsequent in vivo studies.

3 Genotoxicity

For small molecules, mutagenicity is a very severe development hurdle, since gene mutagens are also regarded as potential carcinogens and may cause germ line mutation, a hazard, which is acceptable only under rare circumstances (e.g., late-stage cancer treatment). Therefore, a hazard for mutagenicity is generally not accepted for drugs. As a consequence, evaluation of the mutagenic potential is often done very early in the candidate screening process. Fortunately, for this endpoint, various in silico tools are available which allow a relative reliable prediction, especially when combining two complementary QSAR systems, one a rule-based expert system (e.g., Derek Nexus, ToxTree) and the other a statistical-based system (e.g., MultiCase, Sarah Nexus, TopKat, Leadscope Model Applier) (Serafimova et al. 2010). The sensitivity and negative predictivity of these systems has reached a level that they are now accepted by regulatory agency for the prediction of the absence of a mutagenic potential in case of impurities (Valerio and Cross 2012; Sutter et al. 2013.). Such methods are also suitable for screening in the early research phase, where high throughput is needed.

Since, for regulatory assessment, a *Salmonella typhimurium* reverse mutation assay with five bacterial strains ("Ames test") is mandatory before FiM, in vitro screening of the different candidates in downsized variants of the regulatory assay is frequently performed. There are several screening formats of the Ames test offering higher throughput and less substance need than the regulatory test performed according to OECD guideline (e.g., Mini Ames, Micro Ames, Ames II, and Ames MPF[6] assay) (Escobar et al. 2013). Positive results in the gene mutation assay are likely to be considered to represent a no-go for further development of the drug candidate, as evaluation of the biologic significance of the finding would require extensive in vivo assessment of the mutagenic and carcinogenic potential prior to start of clinical studies. However, as outlined in ICH S2 (R1) (2011), purity of the test substance should be considered in order to exclude impurities as cause of the mutagenicity.

In addition to the bacterial gene mutation, chromosomal damage or recombination may occur resulting in clastogenic and/or aneugenic effects. In regulatory testing, this endpoint has to be evaluated in a mammalian cell genotoxicity assay. Therefore, screening for such effects is also common in the drug discovery process. From the multitude of available in vitro tests, the micronucleus test is the most frequently applied screening assay due to the potential of automation and thus relatively high throughput (Kirsch-Volders et al. 2003). The other well-established mammalian tests – the mouse lymphoma *Tk* gene mutation assay and the metaphase chromosome aberration test – are much more labor intensive and may still be used as pivotal regulatory assays.

Different from a positive result in the gene mutation assay, a positive result in the in vitro mammalian cell assay does not automatically result in the termination of

[6] Microplate format

a promising drug candidate. The reason for such a different assessment is the higher rate of false-positive results compared to the Ames test but also the fact that the effects determined in the micronucleus assay may be caused by mechanisms which have a no-effect threshold, i.e., the biological relevance of the in vitro finding may be worth checking in appropriate in vivo assays (Thybaud et al. 2007). In case of regulatory testing, in order to rule out a relevant genotoxic effect observed in vitro, two different assays examining two different tissues exposed in vivo should be negative of a relevant genotoxic effect. Examples for such assays are the in vivo micronucleus test in rodent bone marrow or peripheral blood combined with the Comet assay in the liver detecting DNA strand breaks. These assays may be integrated into a repeat-dose toxicity study, when adequately high exposure is reached. Such an integration represents a major reduction of animal use compared to stand-alone tests. There are also other assays, which may be appropriate depending on the type of damage to be checked (ICH S2 (R1) 2011). Such in vivo assays include UDS[7] test, SCE[8] test, covalent binding to DNA, and transgenic mouse models (Cimino 2006). Due to the fact, that these assays are rather used as stand-alone follow-up tests, they do not play a role as screening assays. In contrast, there are currently new methods for in vivo detection of point mutations under development, which may allow integration of this endpoint into systemic toxicity studies in order to put into perspective a positive Ames mutagenicity test (Dobrovolsky et al. 2010). Such an assay could then also be integrated into early in vivo screening studies.

4 General Toxicity

The initial test giving some idea of general toxicity is a cytotoxicity screen performed early in drug screening (Ekwall and Johansson 1980). It may include testing of quiescent cells compared to rapidly dividing cells in order to get an idea on the mechanism of action. The cell types may include human and animal cell lines as animal primary cells harvested from different organs. Cell line assays are available for high-throughput screening (Xia et al. 2008). Such data give a rough orientation on general toxicity and may be useful for early screening of specific organ toxicities (see following sections). When the EC_{50} for cytotoxicity is several orders of magnitude higher than the EC_{50} of the intended primary pharmacodynamics effect in a relevant cell model, this may be interpreted as indicator of good tolerability – when a low window is identified, this may hint toward significant development hurdles.

With regard to a holistic evaluation with well-established extrapolation to human, the in vivo repeat-dose toxicity study is still the “gold standard” for general toxicity testing. Such in vivo screening studies are usually performed during lead

[7] Unscheduled DNA synthesis

[8] Sister chromatid exchange

optimization phase with a small number of candidates. The species selection is based on the same principles as the selection of the species for the pivotal studies. There are no fixed rules on animal number, dose selection, study parameter selection, and treatment duration. In addition, whether only a rodent species or a rodent and a non-rodent species are used for screening may be decided case by base based on prior knowledge on the compound class, the pharmacological mode of action, and the level of concern related to the individual drug candidate. Since the pilot repeat-dose studies may also be used as dose-range-finding studies for the pivotal studies, testing in the species also intended for the pivotal repeat-dose toxicity studies for FiM at dose levels supporting dose rationale of the pivotal studies helps limiting the number of animals used for the testing program to a minimum. Sometimes, availability of test substance may be a factor to be taken into account, when planning the studies.

These pilot studies are mostly performed as short-term repeat-dose toxicity studies with treatment duration of 3–14 days. Analysis of larger data sets of small-molecule drugs showed that 14-day toxicity studies in a rodent and a non-rodent species are best suited to reduce attrition rate during GLP phase (Roberts et al. 2014). However, there are still relevant adverse effects only found after longer-term treatment with most being detected in the 4-week repeat-dose toxicity studies (Olsen et al. 2000; Greaves et al. 2004; Tamaki et al. 2013), so that studies with 4 weeks of dosing are the best approach for the pivotal GLP toxicity studies enabling first-in-man studies, although in principle, a 2-week duration would be sufficient according to minimum standards defined in ICH M3 (R2).

When using the pilot repeat-dose toxicity studies also as dose-range-finding studies for the pivotal GLP studies, the high dose should be selected based on the principles described in ICH M3 (R2). Criteria are the reaching of the maximum tolerated dose (MTD) or the maximum feasible dose (MFD), e.g., characterized by exposure saturation, testing of a limit dose (generally 1,000 mg/kg for repeat-dose), or testing of a high multiple of the anticipated human dose (50 times the intended human therapeutic dose) (Buckley and Dorato 2009). For definition of MTD, body weight development may be used in addition to clinical signs (Chapman et al. 2013). Margins of exposure for general toxicity studies may be estimated based on the human equivalent dose (HED) as defined by the FDA guidance for industry (2005) or based on fraction unbound of the AUC.[9]

Study design in these studies should be tailored to the compound. The parameters tested depend on the endpoints to be covered. Histopathology should at least include the vital organs like the heart, lungs, liver, kidneys, and brain, some endocrine organs, some lymphatic tissue, and the administration site (e.g., gastro-intestinal tract for oral dosing and veins for i.v. dosing). Animal number in rodents should be high enough to allow adequate sampling for toxicokinetics evaluation. In non-rodents, 1 to 2 animals per group may be sufficient. In cases, where the

[9] Area under the curve

dose–response relationship in vivo is not well known (e.g., first-in-class compound), a stepwise approach may be helpful (Smith et al. 2005).

5 Cardiovascular Effects and Cardiotoxicity

Cardiovascular toxicity is one of the major reasons for failure of a compound in drug development. In this context, drug-induced prolongation of the QT interval in the electrocardiogram (ECG) is of specific concern, since it may result in torsade de pointes, which may be fatal. Torsade de pointes occurred for several drugs on the market and resulted in the withdrawal of some of them (Yap and Camm 2003). Therefore, regulatory guidelines were developed requiring a thorough evaluation for this risk, resulting in ICH S7B (2005) for nonclinical testing and ICH E14 (2005) for clinical testing. Regulatory nonclinical testing includes evaluation of the impact on ionic currents in an appropriate cellular systems, action potential parameters in vitro or in vivo, and ECG measurement in an appropriate animal model. As mandatory ionic current, the current through the I_{Kr}[10] channel protein (encoded by hERG[11]) is listed in ICH S7B.

There are some computational tools for prediction of hERG blocking and potential QT prolongation (Taboureau and Jørgensen 2011; Valerio et al. 2013). However, these systems are still evolving and prediction is limited because of the complexity of the myocardium. Therefore, in silico approaches for cardiac repolarization are not as common in drug discovery as for genotoxicity (Lindgren et al. 2008; Raschi et al. 2009).

Because of the importance of the cardiovascular liability in drug development, early screening for binding to the I_{Kr} channel protein as well as early screening for I_{Kr} channel blocking potential is commonly done (Lindgren et al. 2008). Therefore, automated miniaturized hERG assays were developed for early screening allowing relatively high throughput (Hancox et al. 2008; Dunlop et al. 2008). In case of a small therapeutic window for hERG blocking, further evaluation in more complex models such as in vitro action potential assay, e.g., in Purkinje fibers, or an in vivo study in relevant animal models (guinea pig or non-rodent model) may be needed to evaluate the relevance of the finding, before terminating a promising candidate because of this liability. Because of the size, the guinea pig is a preferred model in the early research phase, whereas pivotal in vivo studies are generally done in non-rodents, most commonly the dogs or alternatively in minipigs or monkeys (Yao et al. 2008).

In addition to the I_{Kr} channel, the interaction with other ionic channels also plays an important role for action potential repolarization and should therefore also be considered as part of the screening strategy before start of full nonclinical development. Such channels include the sodium channel I_{Na+}, the L-type calcium channel

[10] "Rapid" delayed rectifier potassium current

[11] Human ether-à-go-go-related gene

I_{Ca++}, and additional potassium channels such as I_{Ks} [12] and $I_{Kto.}$ [13] For these channels, automated patch-clamping screening methods are available (Moeller and Witchel 2011). Human cardiomyocytes derived from stem cells may provide a promising early screening tool for cardiomyocyte function (Himmel 2013). In addition to in vitro cell-based models, zebrafish larvae can be used for early screening in a more complex system. However, because of the route of exposure (transdermal), this test system may be less sensitive than the cell-based methods (Sukardi et al. 2011).

Furthermore, other cardiovascular effects can be monitored in vitro in isolated tissues or organs such as isolated muscle preparations, vascular preparations, or isolated perfused organs such as rat heart using the *Langendorff* technique. With these techniques, cardiomyocyte effects as well as vascular effects may be tested (Ramos and Acosta 2000). Cardiovascular endpoints (e.g., systolic and diastolic blood pressure, cardiac output, left ventricular pressure) may also be included in the in vivo cardiovascular safety studies or in the general toxicity single- or repeat-dose studies by assessing hemodynamic effects via blood pressure monitoring. When looking at best practice, most companies perform an in vivo pilot cardiovascular safety study before moving drug candidates forward to regulatory preclinical testing phase (Lindgren et al. 2008). Use of a non-rodent species for this in vivo study may reduce attrition rate during the GLP phase (Roberts et al. 2014).

Another aspect of cardiac safety screening is the identification of cardiotoxic liabilities, since in addition to functional alterations of the heart, accumulative morphological damage can also lead to termination of candidates. Depending on the level of concern for such findings, screening may be done early by using models enabling relatively high throughput and low compound need, e.g., for anticancer drug classes known for cardiovascular toxicity (Doherty et al. 2013; Mordwinkin et al. 2013). Evaluation of cardiotoxicity can also be integrated in the general toxicity studies for the more advanced candidates by including plasma biomarkers for myocyte damage such as AST,[14] LDH[15], and CK[16] and more specific cardiomyocyte markers such as troponin (Reagan 2010) as well as histopathology of the heart.

6 Central Nervous Effects and Neurotoxicity

Impairment of central nervous functions as well as neurotoxicity may result in the termination of the development of drug candidates depending on the indication and the severity of the findings. Therefore, screening for central nervous effects is often

[12] "Slow" delayed rectifier potassium current (KLQT1/minK protein form the channel)

[13] Transient outward potassium current

[14] Aspartate aminotransferase

[15] Lactate dehydrogenase

[16] Creatine kinase

included in the candidate selection. The extent of screening may also be guided by the potential for off-target binding as described in Sect. 2.

In vitro evaluation with regard to neurotoxicity is limited because of the complexity of the central nervous system – electrophysiological and chemical interactions between cells and heterogeneous cell types in the brain (different neurons, ganglion cells, and glia cells). There are several options for early screening, but the tests mostly focus on one aspect of central nervous toxicity. One can screen for blood–brain-barrier penetration (Culot et al. 2008), since drugs not penetrating the blood–brain barrier only have a low potential to cause neurotoxicity. In addition, there are models for testing of electrophysiological activity (McConnell et al. 2012). Furthermore, various brain cell types are available as cell cultures. Such models can also be used for mechanistic evaluation of central nervous effects (Gad 2000; Harry and Tiffany-Castiglioni 2005). There are also methods to test interaction between different cells (Frimat et al. 2010). In the future, such screening may be performed with various human cell types derived from induced pluripotent stem cells, thus eliminating issues of species specificity. Although first data are available, such systems are not yet considered ready for routine use (Scott et al. 2013).

In addition to in vitro systems, zebrafish larvae can be used to study behavioral effects as well as cell damage potential. Automated screening can be done with relatively high throughput. Therefore, use of zebrafish larvae allows a relatively broad testing in early drug discovery, but evaluation of the relevance of the data is still ongoing (Parng et al. 2007; Winter et al. 2008; Sukardi et al. 2011).

Most frequently, central nervous function screening is still done for advanced candidates in vivo in rodents using functional observation battery, Irwin test, or locomotor activity either in dedicated pilot safety pharmacology testing (Castagné et al. 2013) or as integral part of the pilot (=screening) systemic toxicity study. In the general toxicity studies, frequent clinical observations as well as histopathological evaluation of the central and peripheral nervous system also provide information on potential neurotoxicity.

7 Hepatotoxicity

Drug-induced liver injury (DILI) is a major cause of attrition in clinical development, for black box warning and for withdrawal of drugs from the market. Whereas preclinical prediction of clinical cardiovascular effects is well established, prediction of hepatotoxicity based on preclinical models is not as reliable because of the lower concordance of the in vivo animal models with the human findings (Peters 2005). There are both false-negative and false-positive findings in the animals. As there are well-established biomarkers to track liver damage in patients, false-positive, mild-to-moderate liver effects in animals can be followed up in clinical trials. However, some relevant liver toxicity in patients may be missed in nonclinical testing. In addition, the so-called idiosyncratic liver damage having multifactorial etiology may only be detected late in clinical development because of relatively

rare occurrence (Abboud and Kaplowitz 2007; Au et al. 2011). Therefore, finding a reliable preclinical testing strategy for early detection of a risk of human liver toxicity was an important area of research in the last decade.

As human hepatic cells are accessible, in vitro cytotoxicity in human hepatic cells can be used to screen for acute hepatotoxicity (O'Brien et al. 2006). Such data in combination with CYP450 interaction data may help to assess potential hepatotoxicity in the lead optimization phase (Dambach et al. 2005; Persson et al. 2011). In addition, more complex systems can be used such as 3D cultures and liver slices, also allowing more readouts (Gómez-Lechón et al. 2010). However, due to more sophisticated culture requirements, such systems may be more appropriate for mechanistic evaluation in case of a specific concern. In addition, hepatotoxicity may also be investigated in zebrafish larvae using high-throughput screening assay, but screening in embryonic immature zebrafish liver does not have an obvious advantage over testing in mammalian, especially human liver in predicting DILI[17] (Mesens 2015).

The relevance of the findings in the early in vitro screens can be followed up in the general toxicity studies performed with the more advanced candidates. Liver changes may be investigated in life by various well-established plasma biomarkers indicative of hepatocellular damage (e.g., ALT,[18] AST,[19] GDH,[20] SDH[21], and total bile acids) and hepatobiliary alterations (ALP,[22] GGT,[23] total bilirubin, and total bile acids) as well as liver function (total protein, albumin, cholesterol). Relevance of the different markers may be species specific, which has to be taken into account, when planning and evaluating the studies (Boone et al. 2005). The most sensitive marker for potential liver effects in animals is still histopathology, so that the liver should be a routine organ for examination in the pilot systemic toxicity studies.

Mechanistic evaluation using, e.g., toxicogenomics techniques may elucidate the cause of the liver effects (Ruepp et al. 2005; Blomme et al. 2009; Ellinger-Ziegelbauer et al. 2011; Zhang et al. 2012). The liver is one of the most intensely studied organs for toxicogenomics evaluation with vast data set either publicly available or accessible via commercial companies. The samples for such evaluation may be collected in the general toxicity studies; in other cases, dedicated studies allowing examination of time course of changes may be more appropriate.

[17] Drug-induced liver injury
[18] Alanine aminotransferase
[19] Aspartate aminotransferase
[20] Glutamate dehydrogenase
[21] Sorbitol dehydrogenase
[22] Alkaline phosphatase
[23] γ-Glutamyltransferase

8 Renal Function and Nephrotoxicity

The kidneys are an important target for drug-induced toxicity, as they are well perfused and as renal excretion is an important route of drug elimination. As kidneys have a high functional reserve, morphological damage may be advanced before it translates in significant malfunction.

In case of concern, initial screening in the early research phase can be done by in vitro methods like cell culture assays or evaluation of tissue slides (Astashkina et al. 2012). However, due to the complexity of the kidney, functional aspects are not well accessible in vitro (Pfaller and Gstraunthaler 1998). There are several cell lines available for high-throughput nephrotoxicity screening including proximal and distal tubule epithelia, kidney fibroblasts, and glomerular podocytes (Huang et al. 2014).

For more advanced candidates, in vivo evaluation for kidney toxicity can be done in the general toxicity studies with histopathology being a very sensitive technique which often detects mild morphological changes prior to any functional impairment. Whereas traditional serum markers for kidney dysfunction (BUN,[24] Crea[25] – indicators of glomerular function) are relatively insensitive, urinary markers can detect kidney injury in an early stage. In recent years, multiple novel biomarkers were evaluated which allow identification of kidney injury and can not only be used in animal studies but may also be used in clinical studies (Bonventre et al. 2010; Dieterle et al. 2010). Some markers are indicative of damage in different kidney structures, others are very specific for specific cell types. Examples of such markers are kidney injury molecule 1 (KIM-1), cytostatin C, clusterin, β2-microglobulin, GST-α, GST-μ, osteopontin, and renal papillary antigen-1 (RPA-1) (Gautier et al. 2010; Betton et al. 2012). Besides such markers, metabonomics investigation of urine as well as toxicogenomics of the kidney tissue may help in mechanistic examinations (Matheis et al. 2011).

For more detailed functional assessment, renal function tests may be performed. Such tests can be performed as dedicated safety pharmacology studies, e.g., in rats, but such function tests may also be incorporated into general toxicity studies. In order to get adequate urinary samples, use of a metabolic cage is advisable during functional testing, especially when also evaluating electrolytes.

9 Respiratory Function and Pulmonary Toxicity

Some aspects of respiratory function may be tested in isolated organs or lung cell cultures. However, respiratory function can holistically be assessed only in vivo. Frontloading of the actual safety pharmacology function test is not commonly done (Lindgren et al. 2008). The most frequently used method is rodent plethysmography

[24] Blood urea nitrogen

[25] Creatinine

(Hoymann 2007). Monitoring of respiration rate, oxygen saturation, and arterial blood gases may be included in general toxicity studies to provide some indication of pulmonary dysfunction.

With regard to morphological organ damage, histopathology of the lungs in the general toxicity studies is a very sensitive method. In addition, bronchoalveolar lavage may be added in order to have some mechanistic readout (Henderson et al. 1985).

10 Developmental Toxicity

Reproductive toxicity including teratogenicity and embryo-fetal toxicity is important, when drugs are intended for use in a woman of child-bearing potential. According to regulatory guidance, such data are only needed prior to entering longer-term clinical studies or use in larger patient populations in those cases, where adequate contraception can be used. In addition, such effects may often be acceptable for drugs when adequate precautions are taken to hinder the treatment of pregnant women (e.g., adequate labeling, risk-management plan).

However, for drugs in some indications, such effects result in termination of the development (e.g., in fertility control). In such cases, early screening for developmental toxicity may be needed. Reproduction is a very complex process. There was extensive research to identify adequate nonanimal methods to predict reproductive toxicity, mainly because of the need to classify a high number of chemicals. Since reproductive toxicity studies require a high number of animals, in vitro systems were urgently needed. However, some aspects cannot be mimicked in vitro. In silico prediction is currently also not advanced enough to allow meaningful prediction.

Nevertheless, several in vitro screening systems have been developed, which may also have some value in the early screening of drug candidates. These include the rodent whole-embryo culture test and the embryonic stem cell test (Brannen et al. 2011). The mouse embryonic stem cell test (mEST), which has the differentiation of pluripotent stem cells into beating cardiomyocytes as endpoint, has already been validated by ECVAM, but the predictive value is still uncertain, as a second data set did not confirm the initial positive results. For the mEST, a high-throughput screening method was developed. The second test, the rat whole embryonic cell culture (rWEC), allows evaluation of multiple organ systems, but it is labor intensive and time consuming and thus, more suitable for mechanistic investigations than early screening (Van der Laan et al. 2012). In addition, a human embryonic stem cell assay (hEST) called DevTox was developed, which may help in screening some aspects of developmental toxicity (West et al. 2010). Zebrafish larvae may also be used as a screening tool allowing evaluation of some aspects of developmental toxicity (Chapin et al. 2008; Sukardi et al. 2011).

The traditional developmental toxicity assays in rodents and non-rodents are still the relevant assays to predict potential developmental toxicity and teratogenicity, but the assays are quite time consuming and need a rather high number of animals,

so that they do not really qualify for frontloading. In case of high concern, a pilot reproduction toxicity study may be performed before start of the initial GLP regulatory studies, but in general, the standard reproduction toxicity studies including the pilot studies are performed later in the drug development parallel to clinical phase 1 or parallel to phase 2, depending on the need for inclusion of women of child-bearing potential.

11 Carcinogenicity

Carcinogenicity is a significant liability for a drug. The nonclinical assessment of the carcinogenic potential for pharmaceuticals is still based on the 2-year carcinogenicity assay in rodents. There are only few shorter-term assays in genetically modified animals (TgrasH2 and TRP53 knockout) allowing replacement of the mouse 2-year carcinogenicity assay (CHMP/SWP, 2004). The rat assay is still mandatory for drugs. The carcinogenicity assays have to be completed prior to marketing application (ICH M3 (R2) 2009).

There is a high interest to find alternative models allowing prediction of the carcinogenic risk for pharmaceuticals. However, this is difficult with no satisfying solution so far (Jacobs 2005). Hazard identification is relatively easy in case of genotoxic carcinogens (integrated in the in silico tools also used for prediction of genotoxicity; see Sect. 3). For non-genotoxic carcinogens, in silico prediction is still poor.

For chemicals, there exist some early screening strategies on how to create early signals for potential carcinogenic hazard. Ames test and structural alerts may identify DNA-reactive carcinogens and in vitro cell transformation assays (CTA) may detect non-genotoxic carcinogens. The most promising CTA is the Syrian hamster embryo cell transformation assay at pH 7 (SHE_7) (Benigni 2012). However, translation to pharmaceuticals is difficult, as a benefit-risk assessment is needed. As described in a recent review, for many marketed drugs, at least one of the carcinogenicity studies was positive and quite some of the drugs also give evidence of occurrence of human cancer (Brambilla et al. 2012). Whether in vitro screening may be helpful in early candidate selection has to be carefully decided because of the limited value for benefit-risk assessment.

12 Other Nonclinical Safety Aspects

12.1 Phototoxicity

Strategies for evaluation of phototoxic potential are well described in ICH S10 (2013). There are some prerequisites that a drug may cause phototoxicity and/or photoallergy: the compound has to absorb light within the range of natural sunlight

(290–700 nm), it has to generate reactive species following absorption of light, and it has to distribute sufficiently to light-exposed tissues such as the skin and eye. Therefore, the initial evaluation of drug candidates with regard to phototoxicity is measuring light absorption at wavelength between 290 and 700 nm. When a compound has a molecular extinction coefficient (MEC) of greater than 1,000 L/mol/cm, it may need further evaluation (Bauer et al. 2014). An assay for detection of reactive oxygen species (ROS) may be performed, but this test is not very specific (Onoue et al. 2008). The in vitro 3T3 neutral red uptake phototoxicity test (3T3 NRU-PT) is a very well-established test with a high sensitivity, though low specificity (Lynch and Wilcox 2011; Ceridono et al. 2012, also described in OECD guideline Test No. 432 (2004)). This test can easily be used for initial evaluation of drug candidates. Depending on the outcome of the study, additional testing may be needed to check the relevance in vivo, as a positive 3T3 NRU-PT may not translate into in vivo phototoxicity (Schürmann et al. 2014). However, as such data are only needed before start of large clinical trials, such additional examinations (e.g., local lymph node assay, autoradiography study) are rarely performed during research phase.

12.2 Phospholipidosis

Drug-induced phospholipidosis is characterized by an intracellular accumulation of phospholipids and the concurrent development of concentrical lamellar bodies. It is primarily caused by an inhibition of lysosomal phospholipase activity by the drug. It has been reported not only for cationic amphiphilic (lipophilic) drugs mainly in preclinical testing but also for a few drugs in humans. The hallmark feature is the ultrastructural morphology. It can affect almost any tissue, but the lung and the liver are frequently affected in the toxicity studies. The functional human consequences are not well predictable (Reasor and Kacew 2001). Therefore, this finding may delay or even terminate the development of a drug.

As the chemical properties with a potential to cause phospholipidosis are well understood, in silico models are available to predict the potential (Orogo et al. 2012). In addition, there are in vitro models available for screening allowing moderate throughput (Kasahara et al. 2006; Morelli et al. 2006). In addition, there are biomarkers for in-life screening in animals and for testing in humans. Evaluation of peripheral blood leucocytes for morphological signs of phospholipidosis may be used as possible marker. In addition, there are some biomarkers to be tracked in blood (e.g., lysosomal phospholipids) and bronchoalveolar lavage samples (e.g., macrophage function parameters) (Monteith et al. 2006). Depending on the severity of the effect, early lesions may already be found in 2-week repeat-dose toxicity studies, but sometimes, such effects are only detected in longer-term studies.

12.3 Mitochondrial Toxicity

In recent years, mitochondrial dysfunction was identified as one of the factors responsible for toxicities resulting in withdrawal of some drugs from the market. Mitochondria are involved in the maintenance of various important cell functions, most dominantly the production of energy. Drug-induced mitochondrial dysfunction may be caused by several mechanisms such as ROS formation and inhibition of mitochondria replication and can manifest in various tissues such as the heart, muscle, kidney, and liver and may also cause ototoxicity (Wallace 2008).

Mitochondrial toxicity may be difficult to identify by in vitro screening with immortalized cell lines, as they do not depend on mitochondria to produce energy, when grown in glucose-rich environment. Use of galactose in the growth media instead of glucose alters the situation, so that such cell lines also depict damage because of mitochondrial dysfunction. In addition, methods to monitor mitochondrial respiration may be used. For early screening, high-throughput models are available, e.g., by using cell cultures or isolated mitochondria. In addition, a variety of mechanism-based in vitro assays are described. Follow-up in the in vivo animal studies is also feasible with lactic acidosis being the hallmark of mitochondrial insufficiency. In addition, ultrastructural analysis of tissues may be used in animals. In addition, modern clinical imaging techniques such as functional MRT and PET allow noninvasive follow-up in clinical studies (Dykens and Will 2007; Hamilton et al. 2008; Nadanaciva and Will 2011).

12.4 Immunotoxicity

Immunotoxicity has to be adequately addressed during drug development, as modulation of the immune system, either immunosuppression or activation of immune cells, may result in severe adverse effects. As many other systems in the body, the immune system is very complex involving different cell types and multiple functional aspects like cell communication and intracellular reactions (Osborn and Olefsky 2012). Some of the basic aspects of testing are described in ICH S8 (2005). It suggests a tiered approach using routine general toxicity studies as first indicator of a potential concern followed by more specific tests addressing different aspects of immune function. Areas to be investigated in relation to immunotoxicity are humoral immune response and cell-mediated immune response, host resistance and nonspecific immunity, and in addition the potential for autoimmune response and contact hypersensitivity. For all these areas, specific functional tests exist (Guo and White 2010; Decotes 2012).

Because of the complexity of the immune system and the different types of immune responses, in vitro screening methods generally address isolated aspects of immune dysfunction such as T-cell function or macrophage function selected based on concerns related to primary pharmacodynamic effect or the drug candidate.

In most cases, initial assessment of the impact on the immune function is done in the in vivo toxicity screening studies by hematological and histopathological examination. In case of concern because of the mode of action, additional parameters such as immunophenotyping of leukocytes or measurement of immunoglobulins may be included in such studies.

13 Omics Technologies

The neologism omics is a suffix for a series of technologies in life science, where pools of biological molecules are concomitantly analyzed by appropriate technologies. In the field of toxicology, toxicogenomics (i.e., the broad spectrum mostly array-based analysis of gene expression) and metabolomics (i.e., the analysis of metabolites in various body fluids by NMR and/or mass spectroscopy after adequate chromatographical separation) have found their entry. The development of toxicogenomics was largely triggered by the advent of the Human Genome Project (Burczynski et al. 2000). However, the big hope to resolve toxicological issues or even revolutionize toxicology by this technology has not been fulfilled. Despite large investments, predictive toxicology, which describes the identification of toxicities by gene expression analysis ideally in in vitro systems, plays no longer a significant role in early drug development. The main reason is the discrepancy between the in vitro test systems and the whole organism with regard to base-level gene expression and onset of gene expression upon a toxic insult (Pognan 2004). On the other hand, toxicogenomics, incorporated into an in vivo study, can provide valuable insights in the mechanisms of toxicity, particularly if the time course of the expression profile is investigated in such a study (Lühe et al. 2005).

Metabolomics can be regarded as an extension of single-analyte determination in conventional clinical chemistry (e.g., cholesterol, glucose, etc.) where the metabolite profile is broadened by using adequate technologies. The advantage of this technology in contrast to toxicogenomics is its low invasiveness, i.e., the body fluid can be collected over several time points in a study without killing the animal. As such the technology has the potential to identify new biomarkers (e.g., bile acids for cholestasis). Similar to toxicogenomics, the large data set resulting from such approaches prevent its use in early screens. However, inclusion into in vivo studies might provide valuable mechanistic insight. Key for the interpretation of metabolomics studies is the access to reference data sets. The sensitivity of metabolomics versus classical regulatory toxicology has been recently assessed for 500 compounds. Comparable sensitivity to classical toxicology assessment was noted in 75% of the cases, increased sensitivity of metabolomics in 8%, and decreased sensitivity in 18% of the cases, thus proving the potential of this technology (van Ravenzwaay et al. 2014).

14 Exploratory Clinical Screening

In addition to the nonclinical evaluation of the drug candidates, revised regulatory guidelines offer various options to perform exploratory clinical studies, which test only low doses with single or short-term multiple administrations in the subtherapeutic to low therapeutic range (Robinson 2008). Because of the low dose, abbreviated preclinical safety testing can be applied, resulting in a significantly lower compound need compared to conventional programs, as the high dose levels to be tested can be distinctly lower than in the standard safety studies (see ICH M3 (R2) 2009). Such early clinical studies can be used to address pharmacokinetic aspects and also drug targeting, e.g., by using imaging technology such as PET (Rowland 2012).

Such exploratory clinical studies ("phase 0") can be helpful, when they support early decision making on the further development of drug candidates before entering full GLP nonclinical safety evaluation (Buchan 2007; Sugiyama and Yamashita 2011). As the required preclinical program does not support a conventional phase 1 clinical study including clinical safety assessment of multiples of the intended therapeutic dose in humans, the nonclinical regulatory GLP safety program has to be completed prior to proceeding into phase 1 clinical studies. Thus, in case of progression of the program, the amount of nonclinical testing is higher than in standard programs. In addition, timelines may be prolonged, when waiting for the results of the phase 0 studies before continuing development (Karara et al. 2010; Yamane et al. 2013).

15 Accessing and Assessing the Acquired Screening Data

In order to make best use of the screening data acquired during early drug development, adequate database and knowledge management tools are required. Whereas single-point measurements of early in vitro pharmacology studies are relatively easily stored in conventional LIMS databases, the more complex multi-endpoint data of in vivo toxicity studies still awaits a broadly accepted database solution. Some companies have developed their own in-house solutions, while others simply rely on the memory of their toxicologists for individual projects results, but there is generally the perception that a quicker and systemic recovery of the acquired data across projects may lead to an improved assessment of early projects even in the situation of scarce data. The European Innovative Medicines Initiative has addressed this need with its eTOX project. The database developed within this project not only developed controlled vocabularies for the mentioned endpoint but also started to share large data sets on toxicity among the participating companies, which will then be accessible for comparison with new drug candidates (Cases et al. 2014).

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Impact of Biomarkers on Personalized Medicine

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Contents

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Abstract

The field of personalized medicine that involves the use of measuring biomarkers in clinical samples is an area of high interest and one that has tremendous impact on drug development. With the emergence of more sensitive and specific technologies that are now able to be run in clinical settings and the ability to accurately measure biomarkers, there is a need to understand how biomarkers are defined, how they are used in clinical trials, and most importantly how they are used in conjunction with drug treatment. Biomarker approaches have entered into early clinical trials and are increasingly being used to develop new diagnostics that help to differentiate or stratify the likely outcomes of therapeutic intervention. Tremendous efforts have been made to date to discover novel biomarkers for use in clinical practice. Still, the number of markers that make it into clinical practice is rather low. In the next following chapters, we will explain the various classifications of biomarkers, how they are applied, measured, and used in personalized medicine specifically focusing on how they are used in de-risking the 10 plus years drug development process and lastly how they are validated and transformed into companion diagnostic assays.

Keywords

Biomarker · Biomarker classification · Biomarker technologies · Clinical trial · Companion diagnostics (CDx) · Drug development · Patient stratification · Personalized medicine · Personalized treatment · Predictive biomarker · Prognostic biomarker

Abbreviations

510(k)	Premarket notification submission
ADC	Antibody drug conjugates
ALK	Anaplastic lymphoma kinase
B-RAF	Isoform B of rapidly accelerated fibrosarcoma gene
BRCA1	BReast CAncer gene 1
BRCA2	BReast CAncer gene 2
BUN	Blood urea nitrogen
CBER	Center for Biologics Evaluation and Research
CDER	Center for Drug Evaluation and Research
CDRH	Center for Devices and Radiological Health
CDx	Companion diagnostic
CECs	Circulating endothelial cells
CFR	Code of federal regulations

CLIA	Clinical Laboratory Improvement Amendments
CMS	Centers for Medicare & Medicaid Services
CREST	Calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, telangiectasia
CTCs	Circulating tumor cells
DCIS	Ductal carcinoma in situ
DM1	Mertansine, a derivative of maytansine
DNA	Deoxyribonucleic acid
Dx	Diagnostic
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
EML4-ALK	Echinoderm microtubule associated protein-like 4-anaplastic lymphoma kinase
EpCAM	Epithelial cell adhesion molecule
FDA	Food and Drug Administration
FFDCA	Federal Food Drug and Cosmetic Act
GWAS	Genome-Wide Association Studies
HbA1c	Glycosylated hemoglobin
HER2	Human epidermal growth factor receptor 2
ICH	International Conference on Harmonization
IHC	Immunohistochemistry
IUO	Investigational use only
IVD	In vitro diagnostic
KIM-1	Kidney injury molecule-1
lncRNA	Long noncoding RNA
LC–MS	Liquid chromatography–mass spectrometry
LDT	Laboratory developed test
miRNA	MicroRNA
mRNA	Messenger RNA
MRM	Multiple reaction monitoring
MS	Mass spectrometry
ncRNA	Noncoding RNA
NGS	Next-generation sequencing
NIH	National Institutes of Health
NSCLC	Non-small cell lung cancer
PAM50	Signature of 50 genes for subtyping breast cancer
PCA3	Prostate cancer gene 3
PCR	Polymerase chain reaction
PMA	Premarket approval
PoC	Proof of concept
PoM	Proof of mechanism
PSA	Prostate-specific antigen
QMS	Quality management system
RNA	Ribonucleic acid

RNase	Ribonuclease
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
RUO	Research use only
SAGE	Serial analysis of gene expression
SCr	Serum creatinine
SNPs	Single-nucleotide polymorphisms
STRs	Short tandem repeats
TMA	Transcription-mediated amplification

1 Introduction

As far as medical literature dates back, references to "biomarkers" can be found. Most often these biomarkers were associated with something noticeably different in a biological sample from a patient that presented with symptoms. These associated differences were then used for the diagnosis, prognosis, and therapy to treat the disease. A well-known example of a biomarker of disease is the analysis of urine. Not only the color and smell but also the sweet taste of urine was used to diagnose and treat diabetic patients. The first official documentation of the term "biomarker" was published by Karpetsky et al. (1977) in a paper describing the potential use of serum ribonuclease levels as a "biomarker" that could be used to aid in diagnosis of patients with multiple myeloma (Eknoyan and Nagy 2005). The term "biomarker" since then and its usage have dramatically increased over the last three decades due to the revolution of molecular biology and medicine, in part because it is now 15 years since the human genome was sequenced but also because the scientific community is now more closely linked by the World Wide Web and large data base consortiums. Through collectively publicized programs like the Cancer Genome Atlas (Kucherlapati et al. 2012; Perou et al. 2012) and Stand Up to Cancer (2015) (www.standup2cancer.org), biomarker initiatives are actively underway identifying numerous biomarkers that may serve as targets for personalized therapy. Increasing efforts to detect and diagnose diseases at the earliest stage are a major driving force behind these biomarker initiatives. However, it is important to mention that there is a substantial amount of work that needs to take place on both the drug development and biomarker fronts to validate a biomarker that is capable of predicting drug response. A bad marker can easily translate into a bad drug, respectively, leading to a wrong treatment.

In 2001, the National Institutes of Health formed a Biomarkers Definitions Working Group to clearly define (Biomarkers Definitions Working Group 2001) what they view as a biomarker since the use of the term "biomarker" can be interpreted differently. Based on this group, a biomarker has been defined as "*a characteristic that is objectively measured and evaluated as an indicator of normal biological processes*, *pathogenic processes*, *or pharmacologic responses to a therapeutic intervention*." In addition to the NIH, the European Medicines Agency (EMA) also came to a consensus on the meaning of what a biomarker is and defined

it as "*a measurable DNA and/or RNA characteristic that is an indicator of normal biologic processes*, *pathogenic processes*, *and/or response to therapeutic or other interventions*." There are several more definitions of biomarkers found within the literature (Strimbu and Tavel 2010), but fortunately there is a general consensus on how biomarkers are being classified and used within the community.

With the emergence of new innovative technologies, the identification and validation of a biomarker is becoming one of the key elements to *personalized medicine*. For example, in cancer, personalized medicine uses specific biomarker information that has been measured from a person's tumor to help diagnose, plan treatment regimens, monitor a treatment response, or aid in determining a prognosis. The goal of personalized medicine is to provide each patient based on his individual biomarker characteristics, beyond the functional diagnosis of his disease, with the right treatment and dose at the right time. Since biomarkers are often expressed differentially in tumor tissues in comparison to healthy tissue, measuring such protein or nucleic acid markers are now proving useful in identifying alternative treatment strategies when the approved first-line therapy has failed. Technologies such as next-generation sequencing (NGS), real-time PCR, and mass spectrometry that used to be only found in academic laboratories are now being used for measuring patient samples in a real-time setting. Such technologies allow for the identification of potential new druggable targets that are specific to the patient's tumor profile. Personalized medicine is seen by some reviewers as more than just tailoring the right therapeutic strategy for the right patient but a way to identify alternative treatment options.

Personalized medicine means something different to patients, physicians, scientists, regulators, and payers. For patients, it is a refined diagnosis of the underlying disease that eliminates the "trial and error" period and expedites a new stratified approach. As not all patients respond favorably to new targeted drugs, research and development scientists heavily rely on biomarker-driven patient stratification to enrich for the population of patients that have the highest chance of a successful drug response. Regulatory bodies, however, view personalized medicine as a way to protect patients demanding that drug development companies investigate in identification of such biomarkers so patients are only given therapies that show some sort of benefit. Lastly insurance companies are demanding greater proof of positive patient outcomes to justify approval, reimbursement, and price on new targeted therapies.

Personalized medicine is here to stay with biomarkers being implemented into early drug development processes and early clinical trials. Scientists are getting better at understanding the demanding processes that are needed to validate, verify, and implement biomarker strategies for all types of drugs ranging from oncology rare diseases to more frequent cardiovascular disorders, while at the same time companion diagnostics are too becoming more of the norm rather than the exception.

2 Biomarker Classification

During the drug development processes, biomarkers are used to determine the success of development milestones. These milestones help with de-risking the drug program as well as de-risking the patient population in which the new drug is to be tested. Biomarkers are used to answer important questions such as "Does this new drug hit the planned target?" "Is this drug safe?" "Is the therapy more effective in one population?" "Does the biomarker predict survival?" "Is this biomarker unique to disease status?" "Does this biomarker guide treatment decisions?" Herein we will describe and answer the aforementioned questions in more detail explaining how biomarkers are used in monitoring the drug development process and clinical responses.

2.1 Does This New Drug Hit the Planned Target?

Prior to and after administering the new drug, biological samples will be collected and used for pharmacodynamic biomarker studies. A pharmacodynamic biomarker shows that a biological response has occurred in a patient who has received a therapeutic intervention, and for which, the magnitude of change of the respective biomarker is linked to the response. Pharmacodynamic biomarkers are largely used for dose finding and often provide decision relevant information that supports the proof of concept (PoC) and/or proof of mechanism (PoM) that is required to be met in order for the drug to continue on through the drug development process. The use of specific pharmacodynamic biomarker in the development of targeted therapies defines important data enabling early go/no-go decisions, selecting combinations of targeted agents, and optimizing schedules of drug combinations. The respective biomarker assays need to provide robust and accurate measurements.

2.2 Is This Drug Safe?

Safety or toxicity biomarkers reflecting a response to treatment are used to detect or monitor adverse effects in a patient receiving a therapeutic intervention. Prior to be administered to humans, several preclinical animal models of different species are used to understand the potential toxicological profile for the new drug. Routine biomarkers such as liver enzymes are measured and are used to determine a suggestive toxicological profile. Urinary kidney injury molecule-1 (KIM-1) is the first biomarker of kidney toxicity qualified by the FDA and EMA and is expected to significantly improve kidney safety monitoring. Traditional biomarkers of renal injury, including serum creatinine (SCr) and blood urea nitrogen (BUN), do not show the sensitivity and/or specificity to adequately detect nephrotoxicity prior to significant loss of renal function. In multiple models of kidney injury, urinary KIM-1 significantly outperformed SCr and BUN (Vaidya et al. 2010). In addition,

these markers are used to identify an adverse reaction at an early stage in a subject receiving drug.

2.3 Is the Therapy More Effective in One Population?

Stratification biomarkers predict efficacy and categorize patients by likelihood of response to a particular treatment, enabling enrichment of patients most likely to respond to therapy. The best described example is again HER2 status for treatment with trastuzumab (Herceptin®). Stratification biomarkers also predict whether a patient develops an adverse reaction to a prescribed drug, enabling enrichment of patients that can be safely treated with a specific drug. For example, P450 variants predict drug metabolism in multiple indications. Many genetic variants have been identified that are known to alter cytochrome P450 (CYP) enzymes and drug receptors, transporters, and targets (Crews et al. 2012). Known mutations in cytochrome P450 are, for example, used to select the starting dose of the anticoagulant warfarin.

2.4 Does the Biomarker Predict Survival?

Prognostic biomarkers are biomarkers that provide information on the risk or likely course of the disease in an individual and the potential overall outcome. Sometimes the terms susceptibility or risk biomarker are used for biomarkers linked to the risk of developing a disease. The BRCA1 and BRCA2 mutations are typical examples in oncology. Germline mutations in the BRCA1 or BRCA2 tumor-suppressor genes are strong predictors of breast and/or ovarian cancer development (Fackenthal and Olopade 2007). In addition, four gene signature panels, the Oncotype DX® (Genomic Health), the MammaPrint® (Agendia), the ProSigna™ (Nanostring), and the Mammostrat® (Clarient Diagnostic) panels, are commonly used to test breast cancer patients. While all four tests are somewhat similar, there are differences: The Oncotype DX® test is used to estimate a woman's risk of recurrence of early-stage, hormone-receptor-positive breast cancer, as well as how likely she is to benefit from chemotherapy after breast cancer surgery. The Oncotype DX® test also is used to estimate a woman's recurrence risk of DCIS (ductal carcinoma in situ) and/or the risk of a new invasive cancer developing in the same breast, as well as how likely she is to benefit from radiation therapy after DCIS surgery. The Oncotype DX® test analyzes the activity of 21 genes. The MammaPrint® test is used to estimate a women's recurrence risk for early-stage breast cancer. The breast cancer can be hormone-receptor positive or hormone-receptor negative. The MammaPrint® test analyzes 70 genes to see how active they are and then calculates either a high-risk or a low-risk recurrence score. MammaPrint® results can help a woman and her doctor make a more informed decision about whether to use chemotherapy to reduce recurrence risk. The Mammostrat® test is used to estimate a woman's risk of recurrence of early-stage, hormone-receptor-positive breast

cancer. The Mammostrat® test measures the levels of five genes in breast cancer cells. These measurements are used to calculate a risk index score. The higher the risk index, the more likely the cancer is to come back. The Prosigna™ test was developed based on the signature of 50 genes. The expression of these PAM50 genes is an accepted standard for subtyping breast cancer and used to classify tumors into 4 different subtypes.

2.5 Is This Biomarker Unique to Disease Status?

Diagnostic biomarkers are biomarkers that help defining a disease in a group of similar diseases. These biomarkers are used to identify a disease, a subtype of a disease, or a specific condition. Typical examples are elevated blood glucose levels and increased glycosylated hemoglobin (HbA1c) as indicators of diabetes.

2.6 Does This Biomarker Guide Treatment Decisions?

Predictive biomarkers are biomarkers that are used for patient stratification, predicting response to a specific treatment, which may increase the chance for a successful treatment. These biomarkers are used to predict whether or not a patient is likely to respond to a specific therapy or treatment regimen. The expression of the drug target is, for example, used in this respect for targeted therapies in oncology. A well-known example is the detection HER2 overexpression in patients suffering from, e.g., breast cancer predicting a likely response to trastuzumab (Herceptin®). Two additional biomarkers that are used to determine treatment decisions for patients that have been diagnosed with advanced stage non-small cell lung cancer are epidermal growth factor receptor (EGFR) and anaplastic lymphoma kinase (ALK). Whether or not these biomarkers contain a mutation or a gene rearrangement determines if patients will be treated with either the drugs erlotinib (Tarceva®) or crizotinib (Xalkori®), respectively.

3 Biomarker Technologies

3.1 Technology Trends and Applications

For the last 10 years, biomarkers (nucleic acid, mutation presence, gene panels/signatures, gene rearrangements, and protein expression patterns) have gained a great deal of attraction and application into clinical trials. This goes in parallel with the increasing number of new targeted approaches in drug discovery and a broad range of new technologies that have helped facilitate these applications. As a consequence of new technological platforms, a significant drop in costs for pharmacogenomics and pharmacogenetic testing has occurred leading to more deep sequencing of patient tumors and large uncombed data sets. In turn, the

diagnostic industry has used these advancements and newly identified gene signatures/panels of biomarkers to show that their technologies are possible for utility in clinical settings.

Emerging technologies such as RNA sequencing, SNP analyses, and multiplexing platforms such as Luminex® and Mesoscale Discovery® have significantly fuelled biomarker identification. With such technologies, routine clinical studies are now able to include biomarker tests that yield the supporting evidence needed to validate the biomarker hypothesis and its use as the surrogate marker for the diagnostic assay. Implementation of these novel technologies and assessments of the clinical relevance and the utility is a time-consuming effort required by pharmaceutical companies. However, there is such an importance and added value to the data generated by these technologies that public–private partnerships have been established, and more collaborations between pharmaceutical and diagnostics companies are happening. Since the biomarker technology landscape is in high flux, the field requires continuous monitoring and changes in regulation of standards set forth by agencies such as the FDA and EMA not only to monitor the quality of the data but also to guarantee consistency within the drug and diagnostic development/implementation processes employed within clinical trials.

The next generation of platforms that are most attentively being watched and provide the most sophisticated and cutting edge advances are the technologies that can deliver the highest specificity and sensitivity but can also detect multiple nucleic acids or proteins simultaneously from a limited amount of starting material. This focus is mainly for clinical use since reproducible biomarker data is needed to support the drug in question. In addition, technologies that have a very streamlined, standardized preanalytic upfront sample handling processes, e.g., supported by point of care devices and less invasive procedures to gain access to human specimens, are being watched. The concept of liquid biopsies is an example of a technology that is still in its infancy but one that will allow closer monitoring of patients over time and can contribute to a better understanding and clinical management of drug resistance in patients with cancer (Pantel and Alix-Panabières 2013).

3.2 Technologies Based on the Molecular Basis of Biomarker

Beside the classical tissue-based immunohistochemistry and in situ hybridization technologies, the current biomarker research field is very much focused on technologies to detect biomarkers in blood and other body fluids. There is especially in cancer patients an urgent need for these less invasive procedures and for technologies that require the least amount of material. Technologies to detect and monitor cancer formation range from the analysis of DNA including mutations, rearrangements, and methylation to mRNA and miRNA to proteins, including altered processing and other modifications. Additional sources for biomarkers are circulating rare cells, e.g., circulating tumor cells (CTCs), immune cell profiles,

exosomes/microvesicles, as well as autoantibodies, cytokines, and metabolites discharged into the different body fluids (Hanash et al. 2011).

The development and implementation of genomics and other "omics" technologies into clinical trials have significantly promoted the identification and validation of novel biomarkers. Pharmacogenomic technologies like NGS are key elements of the emergence of personalized medicine. The progress of technologies generates new knowledge on potential association of genomic biomarkers of health and disease at an unprecedented speed in science: however, translation of this information into clinical tools currently lags behind due to the difficult processing and interpretation of such large data sets. Complex algorithms are being developed and used to support biomarker gene panel signatures that are proving to be not only diagnostic but also prognostic. Also the lack of a clear rationale for the choice of genomic techniques and difficulties in obtaining sufficient samples to properly validate and address the needed clinical correlations lead to non-reproducible and clinically non-validated biomarker data, highlighting the potential of genomics but slowing down the delivery of clinically usable tools (European Medicines Agency, Concept paper on good genomics biomarker practices 2015).

3.3 DNA Biomarkers

Genetic information that is coded within DNA directs the production of proteins required for the cell structure and function of cells over a lifetime. Some authors state that DNA is stable over an individual's lifetime, and biomarkers explicitly representing this stability are termed "DNA biomarkers." Single-nucleotide polymorphisms (SNPs), short tandem repeats (STRs), deletions, insertions, or other variations on the DNA sequence level are among this group. Due to the availability of high-throughput molecular biological facilities, SNPs are the most commonly used type of DNA biomarker. Beside the established role of point mutations in protein-encoding genes as diagnostic or pharmacogenetic biomarker, genetic variation contributes to both disease susceptibility and treatment response. Genome-wide association studies (GWAS) have enabled rapid discovery of genetic variants contributing to the pathogenesis of complex genetic diseases as well as detection of many pharmacogenetic markers. Such pharmacogenetic markers have been successfully used to explain drug exposure and clinical response variability, to calculate the risk for adverse events, to guide genotype-specific dosing, and to explain mechanisms of drug action in case of polymorphic drug target. However, it is fair to say that the vast majority of risk alleles that have been found by GWAS studies since 2005 are still far away from being used for individual disease prognosis or even for drug treatment decisions. A good example is the Factor V Leiden mutation that is consistently found in GWAS studies as being strongly associated with an increased risk of venous thromboembolism, without justifying preventive treatment of mutation carriers with anticoagulants. The driving hope of these major advances in genetic epidemiology is that promotion of personalized medicine will improve medical decision-making (Ziegler et al. 2012).

3.4 RNA Biomarkers

In contrast to the very stable DNA biomarkers, the versatile transcriptome with all its different components like mRNA, microRNA, and short and long noncoding RNAs is the next level on which dynamic changes on the molecular level can occur. Some of the methods used to detect biomarkers at the RNA expression level include quantitative reverse transcription polymerase chain reaction (RT-qPCR), serial analysis of gene expression (SAGE), differential display, bead-based methods, and microfluid card and microarray analysis. Comparative analysis of RNA expression in terms of heat maps, supervised algorithms, and snapshots are eventually linked with diagnosis and prognosis.

The major attraction in transcriptomics as a starting point for biomarker identification is the ability to measure mRNA concentrations of all genes under any condition that allow studying regulation of gene expression at a genome-wide scale. The genome-wide search for mRNA biomarkers is since more than 20 years an established method in different life science fields. In pharmacogenomics mRNA expression levels were successfully applied to establish treatment prediction with specific drugs. In most studies, a number of genes whose expression was influenced by treatment could be identified. Hence the identification of a biomarker pattern consisting of various expressed genes will be more promising than finding standalone single markers. Despite the impressive number of publications aiming on the detection of mRNAs that predict disease progression or indicate the appropriate use of a specific drug treatment, the current FDA list of pharmacogenomic biomarkers in drug labeling does not even count ten different mRNA-based assays.

3.5 microRNA

Instead of the classical analysis of mRNA, the quantitative analysis of microRNA (miRNAs) is more and more used for biomarker identification and establishment. miRNAs are small noncoding RNA molecules with 20–22 nucleotides which are involved in posttranscriptional processing of mRNA. They are able to regulate physiological pathways and metabolic processes and therefore impact the entire cellular physiology, organ development, and tissue differentiation. Most miRNAs are known to be expressed in a physiological-, tissue-, and disease-specific manner. Due to their short length, they are less sensitive to RNase exposure and hence are more stable than the longer mRNA with an average length of 2 kb. It is already proven that miRNAs have the potential in the diagnosis of specific types of cancer. For example, tissue derived from gastrointestinal cancer can be differentiated from non-gastrointestinal cancer tissue by analyzing specific miRNA profiles. As also described for mRNA, the miRNA profile characterization gives insights in the progression of specific diseases or the response to a given therapeutic approach, e.g., in breast cancer, miR-210 levels correlate with sensitivity to trastuzumab (Herceptin®) and miR-125b is predictive of chemoresistance (Roosbroeck et al. 2013).

The expression of miRNAs cannot only be measured in tissue or cell culture samples; they are also present in body fluids, like urine, blood, or even milk. Some of those circulating miRNAs are already known to be specific disease markers, especially for different forms of cancer. In the field of cardiovascular research, several microRNAs in serum have been found to improve the diagnosis of acute coronary syndromes on top of the clinical gold standard high-sensitive cardiac troponin. To be used as a routine biomarker for ACS, the speed of the quantification of microRNAs must be increased. The current PCR-based quantification of miRNAs takes much too long to affect decision-making in acute life-threatening conditions such as acute coronary syndrome and is thus unlikely to replace or even complement cardiac troponin assays.

Some level of caution should be taken into consideration when assessing the usefulness of circulating RNAs as biomarkers, as recent studies report on the importance of the origin of biomarkers and their impact on biomarker specificity. For example, a significant proportion of miRNAs derived from red and white blood cells have been found to be present as contaminants in plasma preparation. In addition, inherent differences in biological samples and the methods of collecting and analyzing them can dramatically affect the detection and quantification of miRNAs and other (noncoding) ncRNAs. To identify true disease-specific circulating RNAs, the approaches used for quantification of these RNAs should be optimized and validated for accurate quantification of circulating RNAs (Roosbroeck et al. 2013).

3.6 lncRNA

Noncoding RNAs with a length of more than 200 nucleotides belong to the group of long noncoding RNAs (lncRNAs). Long noncoding RNAs have only just recently been identified to play a major role in gene regulatory pathways for a wide spectrum of human disease conditions, including multiple cancer models. Presently there are already numerous regulatory (and other) roles for which lncRNAs have been identified to be responsible for, though such roles can be classified as either positive or negative expressions of gene regulation at either one of the transcriptional or posttranscriptional levels (Ayers 2013).

In biomarker research, the group of lncRNAs is coming into focus, especially in cancer research. Due to its regulatory functions, different potential lncRNA biomarker candidates are already available. One of the first identified lncRNAs, H19, is a biomarker for tumors of the esophagus, liver, bladder, and colon and for metastases in the liver. A loss of methylation in its promoter region leads to a strong upregulation of this lncRNA, indicating the presence of tumor tissue. Similar to miRNAs, lncRNAs are also detectible in body fluids, although they are less stable than microRNAs.

One FDA-approved diagnostic assay for prostate cancer is based on the detection of the long ncRNA PCA3 in urine samples of patients at risk for prostate cancer. The Progensa™ PCA3 test from Gen-Probe Inc. uses the transcription-

mediated amplification (TMA™) technology to determine a PCA3 score from male urine. TMA is an isothermal nucleic acid-based method that can amplify RNA or DNA targets a billion-fold in less than 1 h. PCA3 is the first long ncRNA to be used in clinical diagnostic assays, but with the recent developments in the ncRNA world, many more will most likely follow soon.

3.7 Epigenetic Factors

The term "epigenetic" defines all heritable changes in gene expression and chromatin structure that are not coded in the DNA sequence itself. With minor exceptions (T- and B-cells of the immune system), all differentiation processes are triggered and maintained through epigenetic mechanisms. Epigenetic inheritance includes DNA methylation, histone modifications, and RNA-mediated silencing, all of which are essential mechanisms that allow the stable propagation of gene activity states from one generation of cells to the next. Several of these major epigenetic aberrations have been developed into biomarkers. Epigenetic biomarkers can be detected in tissue and in blood as circulating DNA (Greenberg et al. 2012). The exploration of epigenetic biomarkers in cancer for clinical use is a relatively new but rapidly developing field. Applications include screening, diagnosis, classification, surveillance, and targeted therapies. If epigenetic factors are to be effective biomarkers in clinical practice, they must be detectable by noninvasive means and outperform the current gold standard, as is true for all new emerging biomarkers. One of the most exciting cases for the use of epigenetic biomarkers outside oncology was the recent finding that DNA methylation status can predict response to therapy with either methotrexate or blockers of tumor necrosis factor alpha in patients with rheumatoid arthritis (Plant et al. 2014).

3.8 Protein Biomarker

While early work has been strongly focused on nucleic acid-based biomarkers (DNA, SNPs and mRNA expression profiles), recent experience suggests that the utility of these markers as clinically applicable decision tools may generally be limited. Protein biomarkers, which offer a significantly greater degree of differentiated information content, are likely to close this gap. Two types of protein assay platforms are currently applied to discover protein biomarkers and to measure them quantitatively and qualitatively (i.e., to determine the isoform state of a protein such as phosphorylation). It is instructive to point out here that an antibody use is a "fit-for-purpose" approach. For example, the requirement for an ELISA is substantially different from that of immunohistochemistry (IHC) or diagnostic assay versus laboratory assay (Qoronfleh and Lindpaintner (2010), www.ddw-online.com).

3.9 Immunoassays: Direct Use of Antibodies

Immunoaffinity-based assays are the mainstay of testing for proteins. They use antibodies directed against the protein or isoform of interest. Detection of the antibody–antigen (protein) complex provides the quantitative measurement of the amount of antigen present in the sample. A variety of methods are used that vary both by how the antibody and antigen come into proximity of each other to form a complex (based on what the antibody or antigen is fixed to) and by the detection method used to monitor the amount of complex. Western blots are the simplest and most widely used immunoassay method in biomedical research. ELISA is the method most often used in clinical settings (e.g., PSA test). A number of platform technologies offer methods for multiplexed and miniaturized immunoaffinity assays (e.g., Luminex®, Meso Scale Discovery®, and PerkinElmer®). Development of antibody-based assays is a time-consuming, resource-intensive effort and frequently hampered by cross-reactivity to other antigens. Moreover, results from immunoassays often do not discriminate among closely related forms of specific proteins. Currently, commercial tests are available for several hundreds of different proteins (using various methodologies); custom-immunoaffinity assay services for others are provided by a number of specialty providers.

3.10 Mass Spectrometry Assays

One of the major challenges facing the emerging field of protein biomarkers is the fact that many biomedical relevant biomarkers are present at very low abundance in human samples. The immunoaffinity LC–MS/MS approach has been specifically devised to address the analytical challenge imposed by the tremendous dynamic range of protein biomarkers, especially in biofluids. For instance, serum or plasma analytes of interest are first enriched in the sample using immuno-based approaches, followed by mass spectrometry-based further characterization. An example of this approach is the design and validation of an immunoaffinity LC–MS/MS assay for the quantification of a collagen type II neoepitope peptide in human urine as a biomarker of osteoarthritis (Nemirovskiy et al. 2010).

While mass spectrometry has been widely used over the years for hypothesis-free detection of protein biomarkers, its application has been impeded by lack of sensitivity and the nonquantitative nature of the tests. More recently, a variant of the technology commonly referred to as peptide MRM (multiple reaction monitoring) is gaining importance as a more quantitative variant for protein biomarker measurements of this platform. Peptide MRM can combine the high selectivity and specificity of mass spectrometry for the protein of interest with impressive quantitative accuracy and dynamic range. Quantitation obtained by this method is based on the peak area for the mass spectra data of the analyte relative to a known quantity of an isotope-labeled standard. The peak area can be used to provide relative quantitation (similar to most immunoaffinity assays) or absolute quantitation (protein concentration). Proteins at low abundance levels in samples will

require a method to enrich for the protein(s) of interest. One such enrichment method is immuno-enrichment with antibodies to the protein or the peptides, another one is the immune-adsorption-based depletion of the sample of abundant protein species. Thus, even mass spectrometry is highly dependent on antibody technology. Indeed, the marriage of the two approaches is fast emerging as one of the most powerful approaches in biomarker research.

3.11 Autoantibodies

An autoantibody is defined as an antibody that an organism produces against the individual's own proteins originating from cells or cell components. Autoantibodies can be used as highly sensitive and specific biomarkers, and their occurrence in blood offers easy access and cost-effective assays. Autoantibodies appear often in quite early disease states and can therefore be used for a respective early diagnosis. The use of immunofluorescence techniques, in which tissue culture cells were used as antigen substrate for detecting autoantibodies, led to the detection of autoantibodies in various autoimmune diseases, including systemic lupus erythematous, scleroderma, dermatomyositis, and mixed connective tissue disease. Certain autoantibodies produce distinct patterns of staining because they react with specific organelles in the nucleoplasm, nuclear membrane, nucleolus, or cytoplasm. An outstanding example is an autoantibody in the CREST (calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia) subset of scleroderma, in which immunostaining revealed a limited number of dots in the nucleoplasm of interphase cells but a total redistribution of these dots to the centromeric regions of condensed chromosomes of cells in mitosis. It became clear that there were multiple autoantibodies of different specificities in any individual autoimmune disease, a few autoantibodies were disease specific, and different autoantibody profiles were associated with different diseases. Such profiles of autoantibodies now serve as diagnostic biomarkers in autoimmune diseases. Their clinical application has, however, been hindered by low sensitivity, specificity, and low predictive value scores. These scores have been shown to improve when panels of multiple diagnostic autoantibody biomarkers are used. A five-marker biomarker panel has been shown to increase the sensitivity of prostate cancer diagnosis to 95% as compared with 12.2% for prostate-specific antigen alone (Zaenker and Ziman 2013).

3.12 Exosomes, Microvesicles

Exosomes and micro-vesicles can be used as biomarkers in translational and personalized medicine. Exosomes are small micro-vesicles which are secreted into different body fluids, e.g., blood, urine, or cerebrospinal fluid by a variety of cells. The expression of proteins in the membrane of these microvesicles and the cytoplasmic RNA and protein content facilitate the allocation to the cell of origin in

healthy volunteers as well as patients. Isolation and molecular profiling of exosomes can provide evidence for the existence of a therapeutic target and therefore be used for patient stratification. Exosomes are on the other hand a valuable source for miRNAs.

3.13 Rare Cells, Immune Cells

A large variety of technologies are under development to capture rare cells of different origin from the blood stream. Molecular characterization of circulating rare cells allows a prediction with respect to the prognosis of the course of a disease as well as the effectiveness of therapeutic interventions. Capturing fetal cells in the bloodstream of pregnant women enables the diagnosis of genetic diseases of the unborn avoiding a risky amniocentesis. Circulating endothelial cells (CECs) can be separated into endothelial progenitor cells and detached mature cells, the latter being indicative for a damage of the endothelial lining. Endothelial progenitor cells reflect a sustained vascularization capability and are suspected to be a marker of angiogenesis and tumor growth in cancer patients.

The increasing number of treatment options under development for patients with metastatic cancer creates an accompanying need for biomarkers to determine whether the tumor will be responsive to the intended therapy, to monitor early response to treatment, and to anticipate emerging drug resistance. Ideally, these biomarkers would be obtained by minimally invasive means to allow serial sampling and to enable quantitative real-time molecular analyses of tumor heterogeneity and evolution as well as drug responsiveness. Molecular characterization of CTCs captured from the blood stream or other body fluids may fulfill this need. The well-known heterogeneity of the primary tumor and metastasis as well as the respective CTCs shedded into the bloodstream determines the need for analysis of multiple CTCs. The current standard for CTC detection is the FDA-approved CellSearch® system by Veridex LLC. This system captures CTCs out of a single blood draw of 7.5 mL based on membrane expression of EpCAM and intracellular cytokeratins. The number of tumor cells circulating in the bloodstream of metastatic cancer patients has been shown to correlate with overall survival (Cristofanilli et al. 2004). As metastasis is the main cause of death in cancer patients, CTCs are seen as a prerequisite for distant metastasis.

4 De-risking Drug Development

Biomarkers are now used in drug discovery and development processes and are proving to be both useful and beneficial in translational and early experimental studies. Biomarkers are incorporated as soon as possible starting with preclinical animal studies to determine and measure pharmacodynamic and safety/toxicity biomarkers. These biomarker assays are then transferred if possible to first-in-man phase I clinical studies. Moreover, depending on the human material that is

collected or archived at the time of diagnosis, the drug developers apply as many high-value information-generating technologies to gleam as much data as possible. Next-generation sequencing (NGS) is now being applied in a retrospective manner to allow the drug development companies more options for the discovery of additional biomarker/gene signature panels that can be used to further determine response, resistance, or patient populations that will most likely benefit. Using prospective or retrospective analyses of pharmacodynamics markers in phase I trials are giving drug developers the window to access the proof of mechanism/concept in limited number of patients. These data interpretations are just one example of how a drug program can be de-risked very early. Predictive markers as well as stratification markers also are being tested in early phase I studies. These studies confirm the biomarker hypothesis. Collectively any biomarker data that can be generated in early phase I/II clinical studies is the ideal way a drug program can be de-risked or refined for greater success.

New classes of drugs that are accelerating personalized medicine are antibody drug conjugates (ADC) that are composed of an antibody that is linked to a drug toxophore. These ADCs are routinely tested in phase I studies in combination with a biomarker assay as the biomarker that is to be measured to determine the patient population to be used in is also the drug target. Archived tissue that has been collected at the time of diagnosis or prospectively collected tissue will be tested by an IHC assay to confirm the biomarker expression level and also determine the prevalence of the biomarker in the test population. An example of a newly approved ADC is a combination of trastuzumab® and a chemotherapy drug called DM1. Trastuzumab® finds the cancer cells and delivers the DM1 upon internalization within the cell. In principle this is how all ADCs work. ADCs are the best example of how biomarkers can speed up the drug development process and how personalized medicine can be applied. The processes in which biomarkers are applied to the drug development are outlined in Fig. 1. Briefly, the use of biomarkers starts with the exploration studies in preclinical models and continues all the way through the pivotal phase II/III trials. These data are continually used to monitor and validate the hypothesis that is needed to support the proof of mechanism/proof of concept in the early development phases and allow for go/no-go decisions to be made.

In parallel with the drug development process, the biomarker also has to undergo a series of development and validation steps. In the event that the biomarker is predictive or a diagnostic marker, the biomarker assay that is used to measure the marker as well as the technology that is used has to be verified and validated just like the development of the drug. Ideally, the biomarker assay and drug should be codeveloped and tested in all clinical trial phases as outlined in Fig. 1. Most often, the biomarker assay lags the drug development phases and will require an expedited development of an assay and a partnership with a diagnostic manufacturer.

Biomarkers/drug combinations are being tested in over 13,000 clinical trials (www.clinicaltrials.gov) with the number of trials increasingly each year (National Institutes of Health 2015). However, it is important to point out that both the drug and the biomarker strategies have to be vetted before the drug can be approved

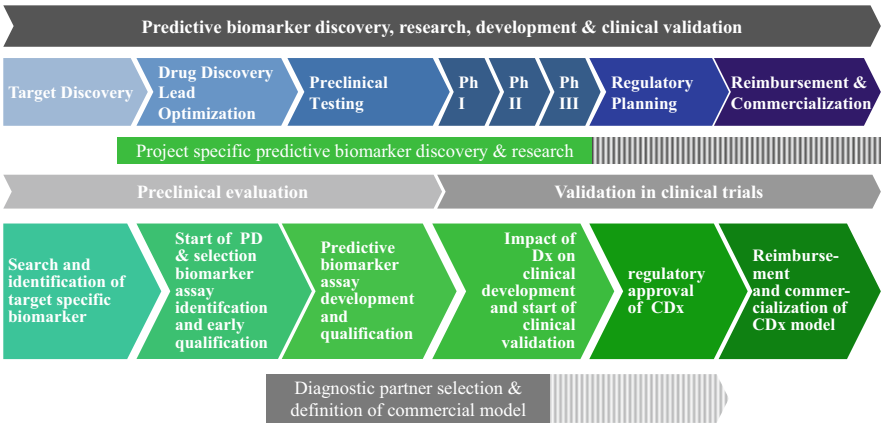

Fig. 1 Overview of the tasks and needs to identify, develop, and validate predictive biomarker along the value chain of drug development. Whereas the target-specific biomarker hypothesis starts to be generated in parallel to the early target validation, the discovery and development of biomarkers can take place along the complete value chain of drug development. Project-specific biomarker assay development starts with the target-specific lead optimization. The development of a diagnostic assay (Dx) which might be used as a companion diagnostic (CDx) in later stages of development should start as soon as the drug candidate and the respective patient population has been selected. Early clinical trials might be used to generate the required data sets for the validation of a diagnostic test to achieve regulatory approval and be used as a CDx

which often is not the case. Recent examples of biomarker/drug programs that did not lead to drug approvals are hENT1 (CP-4126) for treatment of pancreatic cancer and onartuzumab (MetMAb®) for treatment of NSCLC. Even though the biomarker hypothesis seemed to be valid and vetted in the early phase trials, when tested in a randomized phase III pivotal trial, the hypotheses did not yield statistical results with increased survival and overall benefit to BM-positive patients. These trials are devastating to the drug development community and challenge the biomarker/drug development processes. It is still not clear why these programs failed, but programs like these raise the importance of collecting additional biomarker data as early as possible and even after the trials have failed to further de-risk the drug development process or prevent similar failures in other ongoing trials.

5 Biomarkers and Companion Diagnostics

In 2011 the US Food and Drug Administration (FDA) released a guidance document (www.fda.gov, 2015a) that emphasized that all drug development programs should include biomarker measurements. The push to include such biomarkers studies was seen as a way to expedite the drug discovery process, a way to de-risk the treatment population in which the drug is to be administered, and lastly a way to further support the drug mechanism of action. This guidance document in a nut shell upturned the way pharmaceutical companies had been developing new

therapeutic compounds. In turn, the drug development process had to be revamped to include the implementation of validated biomarker assays and in some cases required the pharmaceutical company to start working collaboratively with diagnostic partners. This minor change of including a biomarker assay into a clinical trial has revolutionized and redefined approaches on how to develop therapeutics.

As a result of these new changes, the FDA established a Biomarker Qualification Program to assist with this new guidance and to support the Center for Drug Evaluation and Research's (CDER) work with external scientists and clinicians in developing biomarker assays. The Biomarker Qualification Program provides a framework for the scientific development and regulatory acceptance of biomarker data that are to be used during drug development. Moreover, this program has been integral in facilitating the identification and implementation of new and emerging biomarkers that are to be evaluated and utilized in conjunction with drug programs regulatory decisions/approvals.

Considering that the United States is not the only drug market, the European Union realized the need to standardize drug development across markets. Like the FDA and the Biomarker Qualification Program, Europe and other countries realized the need to rationalize and harmonize the regulations. This was impelled by concerns over rising costs of healthcare, escalation of the cost of R&D, and the need to meet the public expectation that there should be a minimum of delay in making safe and efficacious new treatments available to patients in need. Therefore, in 1990, a meeting was held with representatives of the regulatory agencies and industry associations from Europe, Japan, and the United States to establish the "rules" that the International Conference on Harmonization (ICH) would support. The ICH since then has outlined the required standards for the technical requirements for the registration of pharmaceutical products that are to include biomarkers. The ICH guidance's have evolved over the last three decades to keep current with the technologies and standards surrounding biomarkers. An example of such guidance is ICH 16 which describes the recommendations regarding context, structure, and format of regulatory submissions for qualification of genomic biomarkers (as defined in ICH E15). Briefly, the qualification of a biomarker is a conclusion that, within the stated context of use, the results of the assessment with a biomarker can be relied upon to adequately reflect a biological process, response, or event and supports the use of the biomarker during drug or biotechnology product development, ranging from discovery through post-approval. Through these various agencies in conjunction with the various guidance documents, strict rules have been defined which must be taken into consideration when incorporating a biomarker within a drug development program independent of the country in which these activities are taking place. Special consideration must be taken early within your biomarker and drug development programs to assure that there will be no delay when trying to register your drug/diagnostics within the EU and especially for registration within the United States.

5.1 Biomarkers: Laboratory Developed Test or Companion Diagnostic

It is important to note that not every biomarker that can be measured in an academic or research laboratory can be translated into a diagnostic assay and used in combination with a new therapeutic drug. This is due to the required levels of assay development, verification, and validation data that needs to be generated before the test can be used in a clinical setting to diagnose, provided prognostic value, or determine treatment strategies. Based on where the biomarker assay will be run determines the level of validation and designates the assay as either a laboratory-developed test (LDT) or a companion diagnostic (CDx). An LDT is an in vitro diagnostic test that is manufactured by and used within a single laboratory. LDTs are also sometimes called in-house developed tests or "home brew" tests. Similar to other in vitro diagnostic tests, LDTs are considered "devices," as defined by the Federal Food Drug and Cosmetic Act (FFDCA), and are therefore subject to regulatory oversight. LDTs are regulated by the Centers for Medicare & Medicaid Services (CMS) (2015) through the Clinical Laboratory Improvement Amendments (CLIA). In total, CLIA covers approximately 244,000 laboratories (Centers for Medicare & Medicaid Services, www.cms.gov). Any tests performed on humans are to be performed following CLIA guidelines to ensure accurate and reliable test results. When a laboratory develops a test system such as an LDT in house without receiving FDA clearance or approval, CLIA prohibits the release of any test results prior to the laboratory establishing certain performance characteristics relating to analytical validity for the use of that test system in the laboratory's own environment. The majority of pharmaceutical companies outside of the United States follow these rules when conducting their pivotal trials since the FDA will not allow drugs to be marketed or registered if the CLIA guidelines are not followed and data are not aligned or compliant.

A companion diagnostic is a medical device/in vitro diagnostic (IVD) that provides information that is essential for the safety and effectiveness use of a corresponding therapeutic product. CDx differs from LDTs in that these tests are run in multiple global laboratories. IVD devices range from needles to pacemakers to immunohistochemical (IHC) or polymerase chain reaction (PCR) assays. The term companion diagnostic denotes that a drug is used in conjunction with the readout of the in vitro diagnostic device. Moreover, CDx is used to identify patients who are most likely to benefit from the therapeutic product, identifies the patients likely to be at increased risk for serious adverse reactions as a result of treatment with the therapeutic product, is used to monitor responses to treatment with the therapeutic product for the purpose of adjusting treatment (e.g., schedule, dose, discontinuation) to achieve improved safety or effectiveness, and identifies patients in the population for whom the therapeutic product has been adequately studied to be found safe and effective, i.e., there is insufficient information about the safety and effectiveness of the therapeutic product in any other population. Depending on how the device data will be used dictates the level of design control (inclusion of special controls and documentation of the whole development process) and

validation the device must undergo before being approved by the FDA. As an example, if the data from an IHC assay will be used to decide what treatment a patient will receive, the device must undergo the highest level of testing, validation, and documentation. This device would be considered a class III medical device since it contains the highest risk to a patient. The FDA uses a risk-based approach on deciding the regulatory pathway for IVD companion diagnostic devices. This means that the regulatory pathway will depend on the level of risk to patients, based on the intended use of the IVD companion diagnostic device and the controls necessary to provide a reasonable assurance of safety and effectiveness. Thus, the level of risk together with available controls to mitigate risk will establish whether an IVD companion diagnostic device requires a premarket approval application (PMA, Class III) or a premarket notification submission (510(k), Class II). FDA recommends that sponsors consult early with FDA on the likely regulatory pathway for the IVD companion diagnostic device. Premarket review by FDA will determine whether the IVD companion diagnostic device has adequate performance characteristics for its intended use. As shown in Fig. 2, the three classes of medical devices are listed. The specifics regarding the other level of devices and further information surrounding design control will not be discussed in this chapter, but the relevant information can be found on the FDA website (www.fda.gov, 2015b).

5.2 Laboratory Developed Test and Companion Diagnostic Requirements

The requirements for laboratory-developed tests and companion diagnostics have been very well outlined by CLIA and the FDA, respectively. In general, because LDTs have not been evaluated by the FDA, they must undergo a more lengthy and rigorous validation process by the individual laboratory wishing to implement the new method. The minimum analytical validation standards that need to be measured for an LDT are accuracy, precision, the test sensitivity, and specificity. Additional metrics may be required if the laboratory is accredited by the College of American Pathologist or the Joint Commission. Often the standards set by these different accrediting groups exceed those set by CLIA. In brief, accuracy is the ability of the test to most closely measure the "true" value of a biomarker. The precision of the assay is the reproducibility and repeatability of the test commonly evaluated by performing replication experiments. The results should show a mean value with a statistical significance that does not exceed 15% of the coefficient of variation. The sensitivity of a biomarker assay defines the proportion of actual positive values which are correctly identified as such (e.g., the patients who are precisely identified as being affected by a specific disease). Assays with a very low incidence of false negatives are considered highly sensitive. The specificity of a biomarker assay defines the proportion of negatives which are correctly identified as such (e.g., patients or healthy volunteers who are precisely identified as not being affected by the disease, the assay has been designed for). Assays with a very low incidence of false-positive results are highly specific.

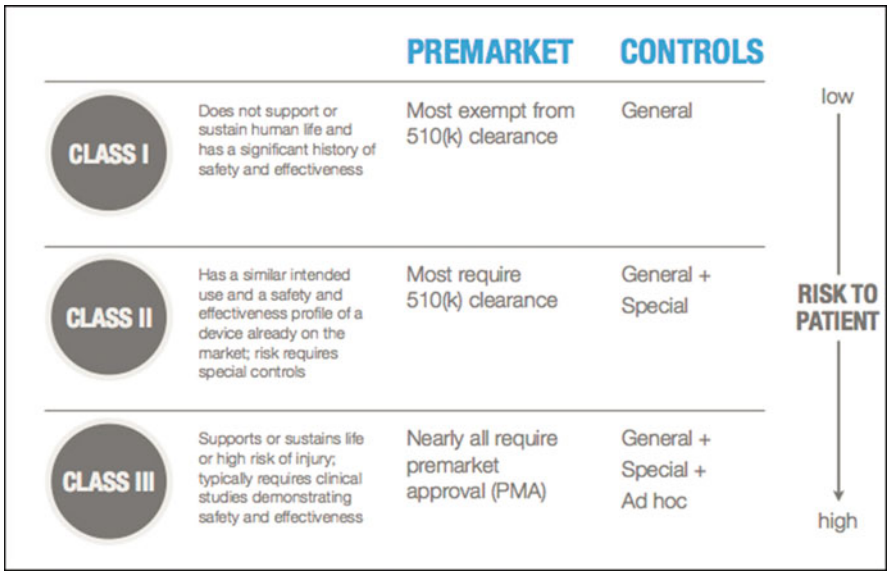

Fig. 2 The FDA has categorized the medical device classes based on risk. Properly classifying a potential medical device will determine the regulatory pathway to be followed to gain FDA approval. Devices are classified as 1, 2, or 3. The time to commercialization can range from 0 days (Class I) to a 90-day FDA review (Class II). In some cases, a multiyear FDA review (Class III) is to be expected. Step-by-step guidelines for classifying a medical device can be found on the FDA CDRH website. All Class II and III medical devices marketed in the United States must be manufactured under the quality systems described in 21 CFR (Code of Federal Regulations) 820. However, it is important to note that the designer of a medical device is considered to be a manufacturer, so the design process must fall within the quality systems. Design controls are critical, especially as the final design is being developed. This is to ensure that medical devices are designed to perform their desired function in a repeatable manner, which is consistent from unit to unit and lot to lot and that changes to the medical device during the design phase are planned, verified, and validated

The requirements for a companion diagnostic include the same analytical validation standards as mentioned above for an LDT but also include that a clinical validation of the biomarker be shown in a clinical trial setting. In some cases, the diagnostic manufacturers have in-house CLIA laboratories where LDTs are developed and use for early phase I/II clinical trial where the assay is used to support the biomarker hypothesis. If the data are compelling enough and the pharmaceutical company decides that the test must be administered globally and in conjunction with the newly developed therapeutic, the diagnostic manufacturer will then use the LDT assay as feasibility data and transfer the assay into a design control mode. Briefly design control consists of the interrelated set of practices and procedures that are incorporated into the design and development process of the assay, i.e., a system with checks and balances. This guarantees quality assurance practices are used and that they are consistent with quality system requirements worldwide. For companion diagnostics that are developed in the United States, the diagnostic

manufacturer must comply with the Code of Federal Regulations, specifically 21 CFR Part 820 which mandates design control and use of a quality management system (QMS) for tracking. Of importance, the time it takes to develop a companion diagnostic can vary depending on the biomarker assay and technology being used. Typically, development times range from 12 to 15 months so planning is quite important when incorporating a CDx into a pivotal clinical trial.

There are various stages of the development of a CDx. As mentioned previously, the LDT or prototype assay can be used early on to support drug trials. This type of assay will be labeled as a research use only (RUO) assay preventing any results to be used in treatment decisions. An investigational use only (IUO) assay is the next phase of the CDx development and builds upon the RUO assay: Using the RUO assay, this assay will be further tested, the reagents will be checked for stability issues, and the conditions to achieve the maximum sensitivity and specificity will be identified. Once all of the various components and conditions of the assay have been tested and identified, the assay is considered locked and at this point the IUO assay cannot be further changed or optimized. The additional testing of the RUO to the IUO is all documented in the QMS and a design history file is created. Over the course of time, if one reagent changes, this change will be documented in the design history file and shows why and when this change occurs. The IUO is the assay that will be used in most pivotal phase III trials.

5.3 Regulatory Authorities

In addition to the design control element of the CDx, there is also a regulatory component of the assay. In order to use the RUO/IUO assay to test human samples that are prospectively collected specifically for testing purpose, an Investigational Device Exemption application must be submitted to the Center for Device and Radiological Health (CDRH) at the FDA. This application consists of manufacturing information, the analytical study design and data (validation), and the clinical trial plan including statistical analysis and cutoff values. Once received at CDRH, there is a 30-day waiting period, and if CDRH has no comments or concerns and the 30 days has passed, the test can be used. If there are concerns surrounding the test or the clinical program, a presubmission packet can be assembled collaboratively between the diagnostic and pharmaceutical companies and sent to CDRH for comments as well requesting a meeting. This packet allows for the diagnostic and pharmaceutical companies to align on expectations and assure that the assay and clinical approach/suggestive cutoff is acceptable to the FDA. The last two additional regulatory elements that are needed are the completion of the premarket approval application which contains all of the diagnostic data collected during the pivotal trial. This data should be filed to CDRH at the same time the drug data is submitted to CDER/CBER which is a very crucial part of the process. The last remaining regulatory element is the postmarket surveillance that occurs after the CDx and drug have been granted approval. This monitoring accesses the safety, effectiveness, and performance of the diagnostic and drug.

There are currently about 20 approved and in-use companion diagnostics. However, the majority of these 20 tests are for the detection of the HER2/new oncogene for treatment with Herceptin. The approval of Herceptin in conjunction with the first approved companion diagnostic IHC test in 1998 has paved the path forward for the search for more novel biomarkers that resemble the same paradigm. A recent success is BRAFV600E companion diagnostic for melanoma. The drug vemurafenib (Zelboraf®) was co-developed with the companion diagnostic to identify the patients that had the mutations BRAFV600E(K,D). This drug received accelerated approvals since the response and survival rates were significantly better than the current standard of care first-line available therapies. The search continues for combination of biomarkers and therapies that can replace the current first-line treatments where often the percentages of patients that respond and have complete remission are relatively low. Personalized medicine is now being applied most often in oncology practices, but in time other diseases such as cardiovascular, infectious disease, rare diseases, etc., too will hopefully have additional companion diagnostic test that help speed up drug development times, identify the correct patient populations, and most importantly provide overall benefit to the patient. In summary, with the increase in emerging technologies and the better understanding of disease heterogeneity, there is a high likelihood for better treatment strategies in the near future.

6 Conclusions

6.1 What Is the Current Impact of Biomarkers on Personalized Medicine?

The expectations regarding the potential of biomarker to transform clinical development and personalized medicine are incredibly high. A lot of biomarker approaches have entered early clinical trials within the last decade. Biomarkers are increasingly being used to develop diagnostics that could help to differentiate, or stratify, the likely outcomes of therapeutic intervention. However, the pace of the uptake of personalized medicine is widely perceived to have been slower than hoped (Milne et al. 2014). Beside a few success stories on companion diagnostics, the impact on personalized treatments and personalized medicine is still limited. The field is still in its early days, but the pharmaceutical as well as the diagnostics industry expects a rapid growth based on the cornerstones that have been laid. Advances in genomics and proteomics help reveal more about the molecular basis of diseases, especially the understanding of disease heterogeneity and drug response.

Tremendous efforts have been made over the past decade to discover novel cancer biomarkers for use in clinical practice as well as developing the required technologies to compliment the clinical setting. Still, a striking discrepancy exists between the effort directed toward biomarker discovery and the number of markers with proven clinical utility. This is not because effort has not been put forth. The list

of Food and Drug Administration (FDA)-approved protein tumor biomarkers in current clinical use is comprised of only 23 proteins (Füzéry et al. 2013) with the majority being to the HER2 oncogene. Extending the approval to include DNA and RNA biomarkers, in the table of pharmacogenomic biomarkers in drug labeling available on the FDA homepage (www.fda.gov, 2015c), 160 entries are found with the vast majority of them applying to the identification of fast or poor drug metabolizers. When broken down to clinical meaningful biomarker, this list ends up with only 25 entries (Majewski and Bernards 2011). It is the case that most biomarkers which are clinically useful were identified through retrospective analysis of clinical trial data. Supporting additional biomarkers should be explored clinically in parallel with the drug development process. In theory this sounds plausible but in reality it is a challenging task. It is quite difficult to develop and validate numerous biomarker assays and technologies and implement them without delaying clinical trials or development timelines. Even with the most sophisticated molecular technologies, there still is a lag with approval of biomarker–drug combinations due to the large amount of data that is generated and the fact that there is a lack of a meaningful clear-cut conclusion. More importantly, the amount of material that is available for testing during clinical trials is often either nonexistent or not compatible with the technology that will be applied for testing. This is not to say that things will always be as they are. With each drug program failure, the community learns lessons and proactively applies new clinical trial strategies to the ongoing trials.

It is fair to say that technology drives discovery and with that leads to better personalized treatment. As mentioned previously, the cost of whole-genome sequencing is no longer cost prohibitive allowing now for patient tumors and genomes to be completely sequenced. Initiatives to sequence 100,000 genomes are being launched by companies and countries, for example, England (2015) (www.genomicsengland.co.uk). The 3-year project, launched by the prime minister of England late 2014, will transform diagnosis and treatment for patients with cancer and rare diseases. The initiative involves collecting and decoding 100,000 human genomes. Projects like these have the potential to transform the future of healthcare. They can improve the prediction and prevention of disease, enable new and more precise diagnostic tests, and allow personalization of drugs and other treatments to specific genetic variants. By participating in initiatives like these, patients will benefit because a conclusive diagnosis can be reached for rare and inherited diseases more quickly. The ultimate benefit will be in the improvement of our knowledge of the influence of genetics on disease and how it is expressed in an individual. Most importantly once this information is available, drug developers can quickly apply this information to their clinical programs and work with diagnostic manufacturers to develop the right tests to detect the biomarkers that are the most meaningful.

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Modeling and Simulation of In Vivo Drug Effects

Jörg Lippert, Rolf Burghaus, Lars Kuepfer, Bart Ploeger, Stephan Schaller, Walter Schmitt, and Stefan Willmann

Contents

Abstract

The concept of a pharmacokinetics–pharmacodynamics (PK/PD) assessment of drug development candidates is well established in pharmaceutical research and development, and PK/PD modeling is common practice in all pharmaceutical companies. A recent analysis (Morgan et al., Drug Discov Today 17(9–10):419–424, 2012) revealed however that insufficient certainty in the integrity of the causal chain of fundamental pharmacological steps from drug dosing through systemic exposure, target tissue exposure, and engagement of molecular target to pharmacological response is still the major driver of failure in phase II of clinical drug development. Despite the rise of molecular biomarkers, ethical, scientific, and practical constraints very often still prevent a direct assessment of each necessary step ultimately leading to an intended drug effect or an unintended adverse reaction. Yet, incomplete investigation of the causality of drug responses is a major risk for translational assessments and the prediction of drug responses in different species or other populations. Mechanism-based modeling and simulation (M&S) offers a means to investigate complex physiological and

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pharmacological processes and to complement experimental data for non-accessible steps in the pharmacological causal chain. With the help of two examples, it is illustrated, what level of physiological detail, state-of-the-art models can represent, how predictive these models are and how mechanism-based approaches can be combined with empirical correlation-based concepts.

Keywords
Mechanism based · PK/PD · Systems biology · Systems pharmacology · Translational

1 Introduction

Along the whole life cycle of a pharmacotherapy – from discovery through clinical development to use of a marketed pharmaceutical product – a profound understanding of the efficacy and safety of the pharmaceutical concept is the basis of responsible decision making. This understanding provides the rationale for informed development decisions and the choice of the right treatment for patient groups and eventually individual patients.

Drug responses can only be understood if the whole causal chain from drug application to drug response or adverse event is considered. The most essential steps in this chain are systemic drug exposure, tissue exposure, target engagement, pharmacodynamic (PD) responses, and finally pharmacological response (Fig. 1).

While systemic drug exposure (i.e., systemic PK) and systemic PD and TD markers can be measured via blood sampling, responses in tissue are usually not experimentally accessible in humans and are a significant challenge in preclinical animal models. Consequently, most PK/PD assessments have to deal with a gap in the information available about the causal chain. A common fallback is the measurement and analysis of PD and TD markers secreted into systemic circulation instead or the measurement of markers in blood cells as surrogate tissue. Unfortunately, both settings may result in erroneous interpretations. Measurements in surrogate tissue are outside the causal chain and eventually arising correlations with pharmacological responses or side effects do not necessarily translate. Measurements of secreted markers are impaired by secretion from other tissues other than those of interest resulting in a similar risk of reduced translatability. In any case, links between PD or TD readouts and pharmacological responses are less reliable if they are indirect or if they rely on empirical correlations only.

The relevance of these theoretical considerations has been addressed by a number of publications on R&D productivity in pharmaceutical industry (Cohen 2008; Empfield and Leeson 2010; Cartwright et al. 2010; Gabrielsson et al. 2011; Paul et al. 2010). An impressive analysis was recently presented by Morgan and colleagues (2012). In an analysis of 44 drug development projects in phase II of clinical development at Pfizer, the authors could demonstrate that in projects in which certainty about target exposure and engagement and pharmacodynamic response was not given prior to phase II, the likelihood of failure was very high

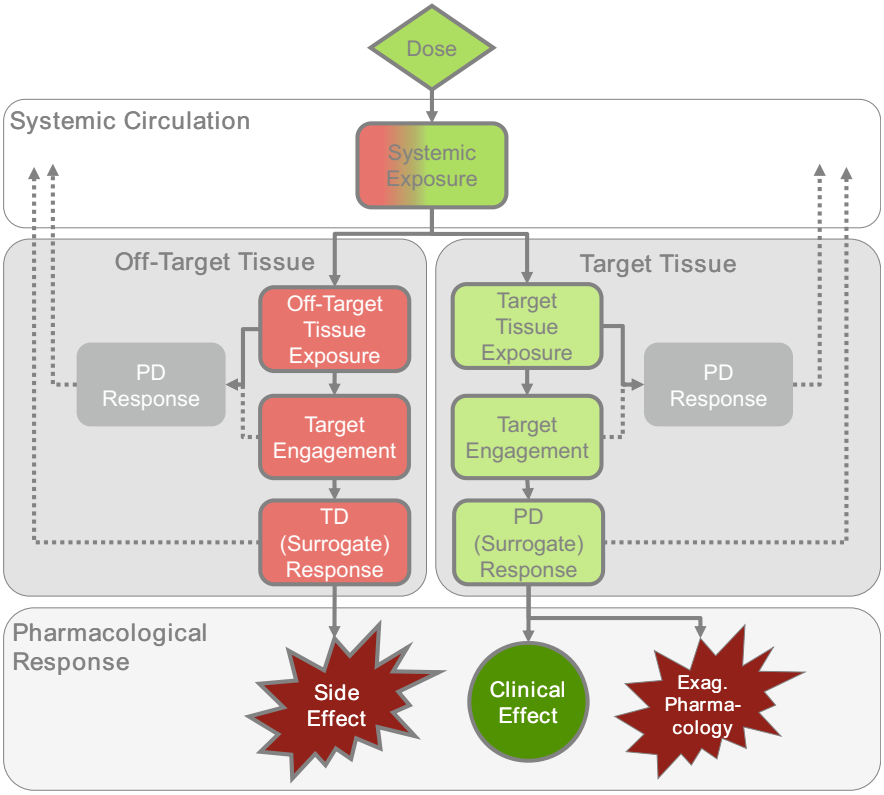

Fig. 1 Drug dose application and absorption may lead to systemic exposure and, in a consecutive step, to exposure in tissue. In both target tissue where a drug effect is intended and off-target tissue where this is not the case, drug exposure may lead to pharmacodynamic (PD) responses not linked to the primary molecular target of the drug. If a PD response is a change in a molecular marker (e.g., the excretion of an endocrine-signaling molecule), this may also impact systemic levels of this marker. If the PD response is linked to a side effect or adverse event, it would also be called toxicodynamic (TD) response. If the molecular target of the drug is engaged, this may again lead to a PD response. In case of a well-established necessary and sufficient causal link to the pharmacological response, i.e., the clinical effect, exaggerated pharmacology, or an unintended side effect, the dynamic response may be considered a surrogate of the pharmacological response

(>80%). By contrast, those projects where sufficient information about target exposure and engagement and pharmacodynamic effect was available prior to phase II showed a very high success rate (12 out 14 projects achieved proof of concept). Morgan and colleagues were well aware of the difficulties to investigate all causal steps with experimental means only and illustrated how M&S contributed to a thorough understanding of the causal chain of pharmacology. In fact, the authors conclude that the analysis of these basic principles has to happen by integrated PK/PD and PBPK modeling but if done diligently has a high chance to pay high dividends of increased R&D productivity.

2 Modeling and Simulation Approaches

Modeling and simulation concepts and techniques have developed independently in several areas of fundamental research in biology as well as in pharmaceutical research and development. Today's armory of M&S methods covers a highly diverse set of approaches from highly detailed representations of molecular-level interactions in signal transduction pathways or metabolic networks typically used for research purposes to abstract statistical models of population-level effects of ethnic, genetic, behavioral, and other covariates on drug exposure in clinical trials used in late-stage clinical development and in regulatory evaluations. While Hodgkin and Huxley (1952) already applied detailed mechanistic computational modeling to study the electric properties of nerve cell axons in the 1940s (a work for which they were awarded the Nobel Prize in medicine in 1963), the use of mechanistic modeling in pharmaceutical R&D is still rather young and developing. In 2011, Sorger and colleagues provided a concise overview of M&S approaches explicitly representing biology and pharmacology, nowadays often called systems pharmacology, in an NIH white paper (Sorger et al. 2011). By contrast, other approaches such as population pharmacokinetics (Ette and Williams 2004) have reached a significant level of maturity documented by industry guidelines from regulatory agencies (US Food and Drug Administration 1999; European Medicines Agency 2007) and recent publications (Romero et al. 2013; Schuck et al. 2015).

The main differences and similarities between mechanistic and abstract statistical M&S approaches can be illustrated by the different approaches to pharmacokinetics (PK), i.e., the concentration-time profiles of a drug in blood, blood plasma, or any other biological matrix resulting from its application to an animal or human volunteer or patient (Fig. 2).

The application of a drug via oral, intravenous, or any other administration route leads to a time-dependent drug concentration profile in the body and each of its compartments, e.g., blood plasma. The so-called PK of a drug depends both on drug properties (physicochemical such as solubility and lipophilicity and biological such as metabolic stability and affinity to protein-binding partners and transporter) and properties of a preclinical species, a volunteer, or a patient (body size and composition, cardiac output, expression levels of enzymes, drug transport, and drug-binding proteins). Classical, abstract approaches to the modeling of PK such as population modeling start when experimental data have been generated in clinical trials (or preclinical experiments) by blood or tissue sampling and analytical determination of drug concentrations. So-called compartmental models (ordinary differential equation (ODE) based) are identified that adequately describe the experimental data. The structures of the models, i.e., the number of compartments (representing parts of the body) and connections between them (representing blood flows, diffusion, and other exchange processes), are chosen in a trial and error manner, such that quantitative parameters can be found that lead to a statistically correct representation of the data. The drug and its properties and the organism are only represented in an abstract and indirect way.

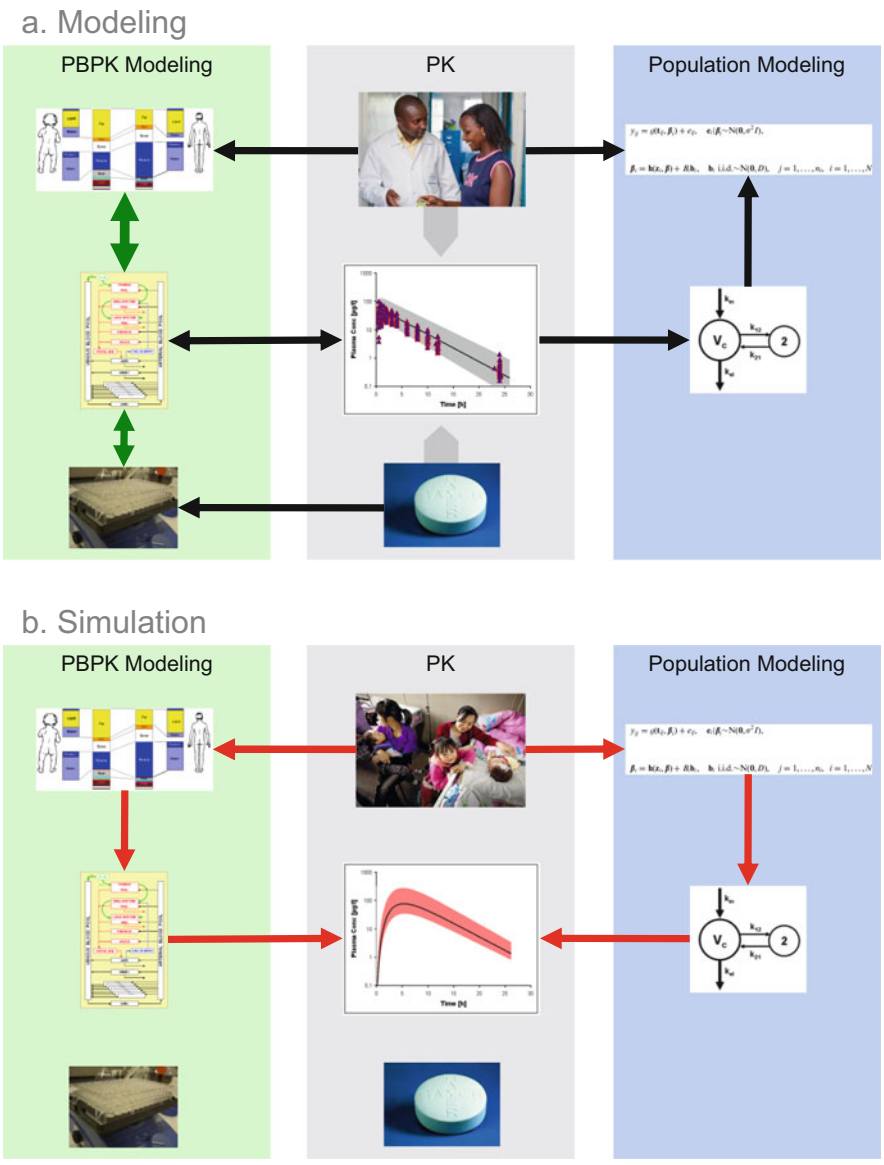

Fig. 2 (**a**) PBPK modeling and population modeling are different approaches to PK modeling. PBPK modeling starts with a prediction based on prior knowledge about drug properties (molecular weight, solubility, lipophilicity, enzymatic stability, protein binding, etc.) and detailed anatomical and physiological knowledge (organ volumes, tissue composition, blood flows, and enzyme and protein expression levels). Numerical model parameters which are explicitly representing chemical and biological properties are adjusted after a comparison with experimental data and only within chemically and physiologically plausible ranges. Population modeling starts with experimental data and identifies abstract compartmental representation of PK where model structures and parameters represent chemistry and biology in an implicit and lumped fashion. The model structure and parameters are a joint statistical representation of the PK properties of a drug and the tested volunteers or patients. (**b**) During simulation, i.e., prediction of new experiments,

By contrast, physiologically based pharmacokinetics (PBPK) modeling starts with an explicit representation of the organism (Willmann et al. 2005). Organs and sub-compartments such as the vascular, the interstitial, and the cellular space are represented individually and connected in way that reflects blood flows and diffusion process or active transport mediated by drug transporters in a 1:1 manner. PBPK models for different animal species and humans differ in the respective model parameters such as organ volumes, blood flow rates, and enzyme and transport protein expression levels. Likewise, models for different human populations such as adults and children also only differ in the respective model parameters representing the dimensions and properties of the bodies (Edginton et al. 2006). Common to all organism parameters is that they are set based on prior information independent of the PK experiments. Typical sources of this information are epidemiological databases such as the National Health and Nutrition Examination Survey (NHANES, http://www.cdc.gov/nchs/nhanes.htm) or the Cancer Genome Anatomy Project (CGAP, http://cgap.nci.nih.gov/) as well as highly specific, dedicated studies of individual properties such as the geographic distribution of different cytochrome P450 2D6 genotypes (Sistonen et al. 2007). In a similar fashion, properties of the drug that is administered are also represented in a 1:1 manner. Solubility, lipophilicity, protein binding, and metabolic stability can be determined in in vitro assays and are represented by individual parameters (Willmann et al. 2005). Consequently, a PBPK model can provide a prediction of the PK of a drug in a given organism before any in vivo experiment has been performed. Once experimental data is available, the PBPK model can be calibrated to better describe the observed PK. As with classical approaches, this is done by adjusting parameter values. The main differences are that organism parameters are usually not touched and parameters representing drug properties are modified only within plausible ranges previously determined in in vitro surrogate assays. In this way, deficiencies of in vitro systems are compensated and, e.g., an in vitro solubility measured in water or intestinal fluid-simulating media is corrected to better reflect in vivo solubility in the gastrointestinal tract.

Simulation, i.e., prediction of new PK experiments, with PBPK models is relying on prior drug-independent information again. For example, organism parameter values representing adult patients are replaced by parameter values describing the different organ volumes and compositions, blood flows, and enzyme expression levels in children to predict PK in pediatrics. In population modeling, new experiments are also simulated. Since parameters are not explicitly representing individual biological or chemical properties but lump drug and organism features,

Fig. 2 (continued) both approaches follow the same procedure. Model parameter values are modified to adjust the model-based representation to new situations such as a new group of patients of different age and body weight and the model is used to calculate the expected PK profiles. In PBPK modeling, the parameters are modified based on prior independent information about patients (e.g., tabulated organ volumes in children and adults) while population modeling uses regression models identified from the PK data for the drug of interest linking so-called covariates such as body weight with one or several of the model parameters

any adjustment of parameters can only be based on correlations between independent properties such as body weight and PK, previously derived from experimental data. So-called covariate models are established that represent these correlations via regression models linking independent properties (e.g., body weight) with abstract model parameters (e.g., volumes of compartments and rate constants for drug clearance from the body). These regression models are then used to predict the model parameterization under the untested, to-be-predicted experimental conditions.

While population modeling is a generally accepted approach in pharmaceutical industry and regulatory agencies for almost two decades and nowadays a mandatory component in regulatory submission packages, PBPK modeling was considered difficult to implement and standardize and consequently limited to in-house and academic applications. This has changed over the last years. For prediction of drug–drug interactions (DDI), PBPK modeling has become a regulatory accepted standard (Wagner et al. 2015) and US FDA itself is using PBPK for applications such as pediatric scaling or translation to other understudied populations and for study design support (Geanacopoulos and Barratt 2015). This development has been largely driven by the availability of commercially available software solutions for PBPK modeling (e.g., PK-Sim®, http://www.systems-biology.com/products/pk-sim.html) that provide databases for prior information on organism properties for various preclinical species from mouse to monkey and important human populations in different geographic regions (Willmann et al. 2007).

3 Representation of Biological and Pharmacological Complexity

The availability of sophisticated M&S tools has enabled the integration of structural scientific knowledge and experimental information from multiple biological levels into unified computational representations that can simulate complex (patho)physiological phenotypes and pharmacological interventions. It is technically no longer a challenge to integrate a subcellular level model of a signal transduction pathway linked to cell proliferation in tumor cells (e.g., MAPK-PI3K pathway) with a model of cell division and apoptosis and to embed it into the affected organ in a whole-body PBPK model representing a drug parent and an active metabolite (Eissing et al. 2011). Limitations to mechanistic modeling result from the lack of available experimental data for quantitative properties of biological and pharmacological entities and processes at individual biological scales and our lack of understanding of the interplay between biological scales, i.e., the interaction between molecular (signaling) cascades and cellular dynamics, between cellular behavior and tissue, etc. In such cases, scientific assumptions need to substitute knowledge and data to enable the establishment of model-based representations. Very often several hypotheses and scenarios (i.e., model structures) are plausible and corresponding model versions have to be established to represent the different possible scenarios. One of the great strengths of M&S is that via simulation these model versions can

be tested against available and future experimental data and can thereby help to rigorously rule out previously plausible assumptions and identify the remaining scenarios.

A previously published model of the glucose–insulin–glucagon regulatory systems (Schaller et al. 2013) is an example of the level of complexity across multiple biological scales that state-of-the-art models can represent. Circulating glucose, a low-molecular-weight sugar, and insulin and glucagon, polypeptide hormones, each need to be represented by PBPK models individually (Fig. 3). Endogenous production and release in the liver and pancreas and exogenous delivery of glucose by food and subcutaneous or intravenous application of glucose, insulin, and glucagon need to be represented as well as uptake and consumption of glucose in the gastrointestinal tract, the liver, muscle and fat cells, and the brain. At organ level, blood and lymph flow, convective and diffusive transport across endothelial cell layer, pinocytosis, receptor-mediated uptake, and FcRn-mediated recycling in endothelial cells are represented (Fig. 4). Insulin receptor internalization, recycling and lysosomal degradation, and the insulin signaling-mediated relocation of GLUT4 insulin-sensitive glucose uptake transporters are further model details.

The different model components were pre-parameterized based on a comprehensive literature research providing typical values for protein expression levels,

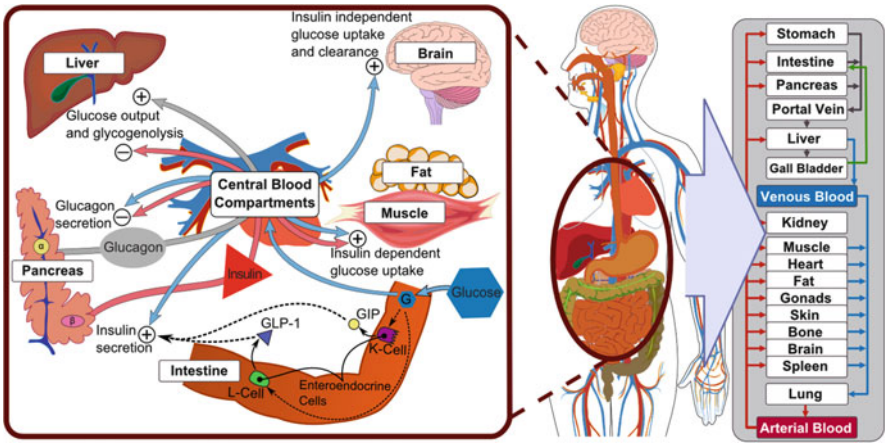

Fig. 3 Overview of the interactions between glucose, insulin, and glucagon (*left panel*): glucose modulates endogenous glucose production, insulin-independent glucose uptake, renal glucose clearance, and pancreatic insulin secretion. During episodes of low glucose concentrations, pancreatic glucagon secretion is stimulated. Insulin maintains glucose homeostasis by insulin-dependent glucose uptake in muscle and adipose tissue. It suppresses endogenous (hepatic) glucose production and activates hepatic glucose uptake. Glucagon, released at low glucose levels, counter-regulates low blood glucose levels and stimulates hepatic glucose production. Integration into a whole-body PBPK model (*right panel*): each physiological organ is represented with several sub-compartments interconnected with convection and diffusion flows (figure modified from Schaller et al. 2013)

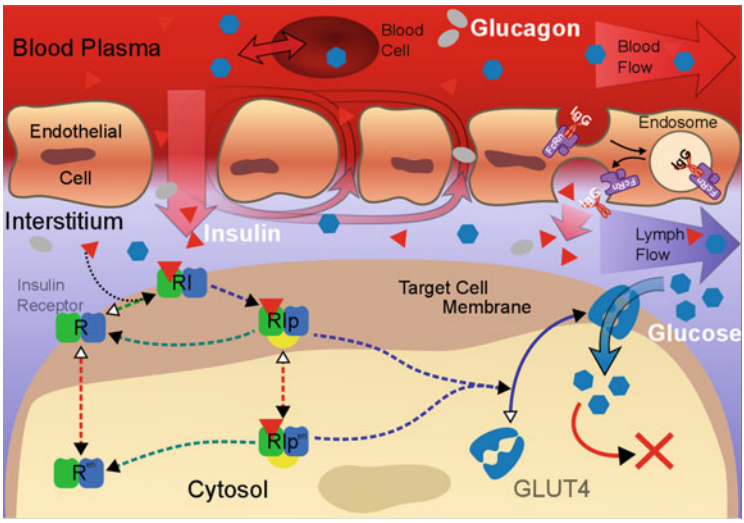

Fig. 4 Sub-organ-level and molecular details represented: organs are represented by five sub-compartments (blood plasma and cells, endothelial endosomes, interstitial, intracellular). Compartments are connected by passive convection, diffusion, and facilitative transports. Distribution of compounds depends on concentration gradients, flow rates, permeability, partition coefficients, transporter properties, and target protein-binding properties. Insulin distribution and its glucoregulatory effects are mediated by insulin receptor (IR)-mediated elimination from the plasma and interstitial space through *trans*-endothelial transport and cellular uptake which triggers molecular signaling in target tissues. Downstream signaling of the insulin receptor in fat and muscle triggers translocation of insulin-sensitive glucose transporter GLUT4, increasing peripheral glucose uptake (figure modified from Schaller et al. 2013)

kinetic constants of transport process, etc. Then, a mean model representing typical behavior under standard glucose and insulin tolerance tests was developed. Parameters were divided into three groups. The first group consists of the parameters assumed to be identical for all volunteers and patients. The second group distinguishes healthy subjects and type 1 diabetics (T1DM) and the third group distinguishes individuals in each group.

The model was then further developed using published clinical trial data with glucose, insulin, and glucagon concentration-time curves at high temporal resolution. El-Khatib and colleagues (2010) studied T1DM patients over several hours and densely sampled all three analytes while they performed insulin, glucose, and glucagon challenges (Fig. 5). The data allowed the adjustment of patient-specific models representing the dynamic responses of the patients to exogenous challenges and meals. A comparison between experimental data and simulated model responses shows an excellent match (Fig. 5).

For each patient, El-Khatib and colleagues repeated the experiment which allowed a rigorous test of the predictive performance of the individualized models. Figure 6 shows a comparison between experimental data and the predicted time

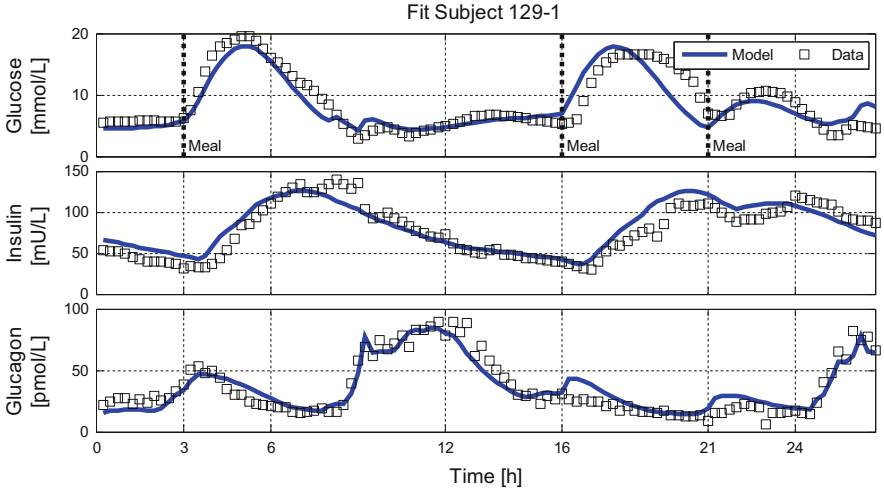

Fig. 5 Simulated fitted trajectories of Subject 129 from the first visit. Simulated trajectories are displayed as *blue lines*; data for comparison is displayed as *black squares*. Displayed are peripheral venous blood plasma concentrations of glucose (*top*), insulin (*center*), and glucagon (*bottom*)

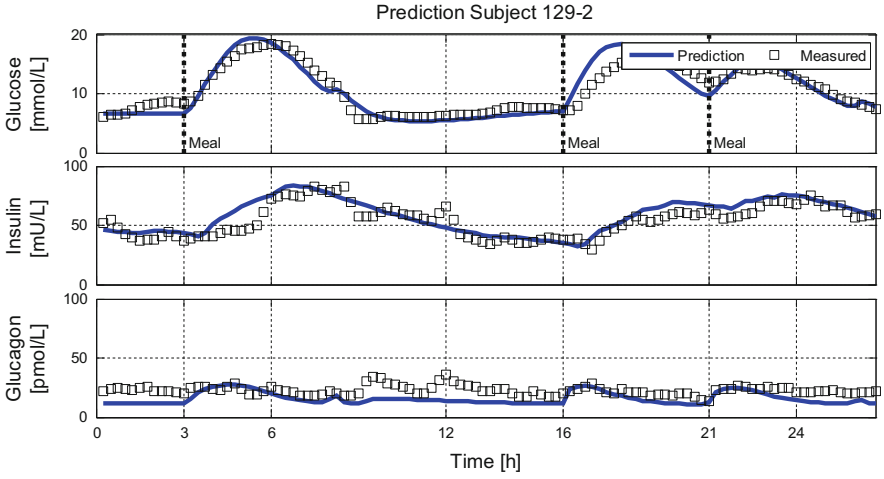

Fig. 6 Simulated predicted trajectories of Subject 129 from the second visit. Simulated trajectories are displayed as *blue lines*; data for comparison is displayed as *black squares*. Displayed are peripheral venous blood plasma concentrations of glucose (*top*), insulin (*center*), and glucagon (*bottom*)

curves based on information about meals and time and dose of each challenge only (same subject as in Fig. 5).

The mechanistic glucose–insulin–glucagon model could be adjusted to all subjects studied by El-Khatib and colleagues (2010) and was capable of capturing

relevant interindividual variability. The predictive performance for subjects' responses to meals and challenges even after several months was very good demonstrating the robustness of the representation and its dynamic behavior. With its size of several hundred ODEs, the model is exemplary for the level of biological detail state-of-the-art modeling approaches are able to capture.

4 Applications of Modeling and Simulation to Situations with Limited Mechanistic Understanding

In pharmaceutical R&D, the knowledge of molecular, physiological, and pharmacological mechanisms is usually limited and important processes may have been identified empirically only. While even intended drug effects are not necessarily fully understood in a mechanistic sense and at all biological scales, the canonical area for knowledge gaps is the field of drug safety. Although toxicological and safety pharmacological testing and monitoring accompany each and every step of drug development and pharmacovigilance continues to detect, collect, and assess safety events after marketing authorization, the causal chain leading to empirically observed but relatively rare adverse drug events is often only understood after intense research and with significant time delays. The importance of drug safety makes it an obvious application area for M&S. In a previously published case study on statin-related myopathy risks (Lippert et al. 2012), it was demonstrated how challenges resulting from knowledge gaps can be overcome by the systematic application of M&S to bridge between existing information linked to drug safety issues.

Statins are generally well-tolerated 3-hydroxy-3-methyl-glutaryl-CoA reductase inhibitors. They are widely used as lipid-lowering treatments with more than 30 million patients worldwide. Mild cases of myopathy occur in around 1–5% of statin-treated patients but only 0.001% develop rhabdomyolysis with a more than tenfold increase in creatine kinase and potentially fatal consequences. Due to the extent of use, hundreds of patients are at risk for severe forms of myopathy, and in genome-wide association studies (GWAS), a single-nucleotide polymorphism (SNP; c.521T → C, p.Val174Ala) in the SLCO1B1 gene encoding the organic anion-transporting polypeptide OATP1B1 has been linked to an increased risk of myopathy after simvastatin treatment. It is also known that myopathy risk is lower at smaller doses or with other statins such as pravastatin.

For drugs such as statins that are substrates of OATP1B1, hepatic uptake by the transport protein can have significant impact on the PK and PBPK models that are able to represent the role of OATP1B1 (Fig. 7).

Once adjusted to simvastatin and pravastatin PK data from subjects with a homozygous OATP1B1 genotype (Fig. 8), the models are able to quantitatively predict the PK in subjects with a heterozygous genotype, a nontrivial task since PK depends on OATP1B1 in a nonlinear fashion (Fig. 8, blue line).

Using prior information about the variability of anatomical (e.g., body weight and organ volumes and composition) and physiological parameters (e.g., blood flow

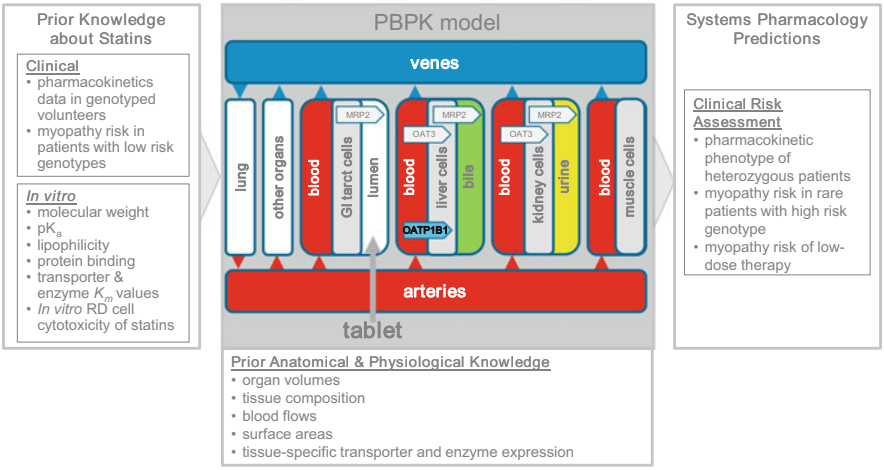

Fig. 7 The PBPK model for statins represents prior information in vitro and in vivo information about statins. In vitro properties of simvastatin and pravastatin are used to pre-parameterize the corresponding models. Clinical PK data for genotyped subjects are used to adjust the models. In combination with in vitro cytotoxicity data and clinical baseline myopathy risks, the PBPK models can be used to simulate clinical incidence rates in populations of high-risk patients underrepresented in small clinical trials due to their low prevalence (modified from Lippert et al. 2012)

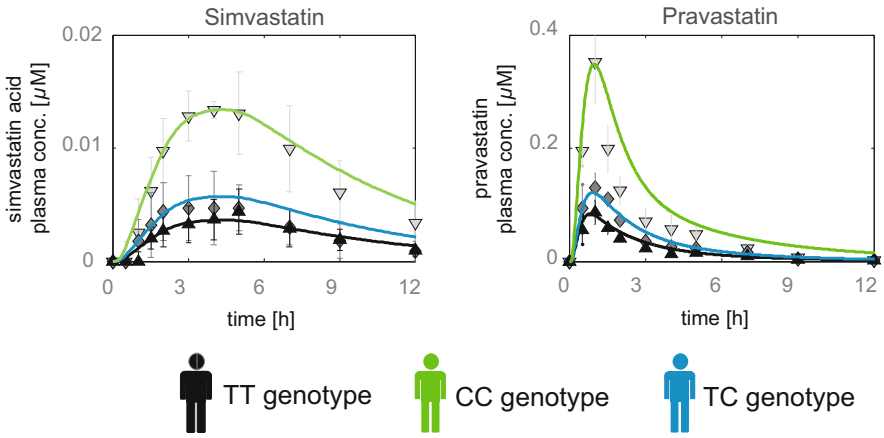

Fig. 8 Simulated simvastatin and pravastatin PK for subjects with different OATP1B1 genotypes. Data (symbols: *black triangles*, TT genotype; *diamonds*, TC genotype; *gray triangles*, CC genotype) for TT and CC genotype were used to adjust the PBPK models. Simulations (*black line*, TT; *blue line*, TC; *green line*, CC) for TC genotype are pure predictions based on the PBPK models for the homozygous genotypes and the assumption of an OATP1B1 activity in the TC subjects equaling the average level of TT and CC activity

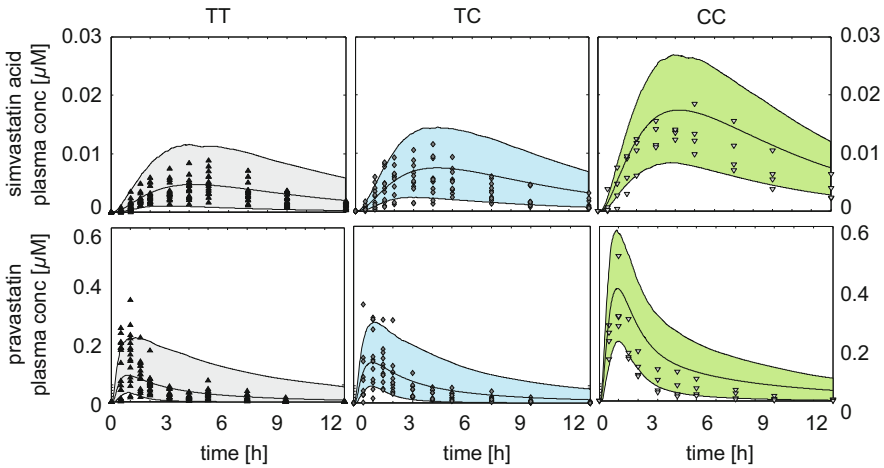

Fig. 9 Plasma concentration-time profiles for simvastatin and pravastatin were simulated for populations stratified by OATP1B1 genotype and compared to experimental data (modified from Lippert et al. 2012)

rates and enzyme and transport protein expression levels), the interindividual variability of plasma concentrations can also be predicted accurately (Fig. 9).

The successful prediction of PK in heterozygous subjects and the correct simulation of PK variability in stratified populations of subjects treated with pravastatin and simvastatin indicate the correct representation of the most relevant PK properties of these statins by the PBPK models.

The CC genotype was linked to an increased risk to develop a myopathy under simvastatin treatment (Link et al. 2008). This is in line with the increased exposure in plasma of patients with the CC genotype (Figs. 7 and 8) and a direct result of the relevance of OATP1B1 for hepatic uptake and clearance from the body (Niemi et al. 2011). The myopathy risk is, however, not directly linked to plasma concentrations of statins. Pravastatin exposure is significantly higher than simvastatin exposure and naïve correction for potency cannot reconcile the differences in myopathy incidence rates alone.

To bridge from well-represented plasma PK behavior to safety events resulting from muscle cell degradation, the PBPK model can be used to simulate mean muscle concentrations obtained in populations stratified by OATP1B1 genotype because muscle is represented explicitly in the PBPK model. The ratio between these predicted muscle concentrations and in vitro potency data (half maximal inhibitory concentrations determined with embryonic rhabdomyosarcoma cells; Kobayashi et al. 2008) provides a toxicodynamic measure. Figure 10 shows the resulting predicted incidence rates for ratio between muscle concentrations and the inhibitory concentration of simvastatin.

A comparison of simulated incidence rates of high toxicodynamic marker levels with clinical myopathy incidence rates in the SEARCH study (Link et al. 2008)

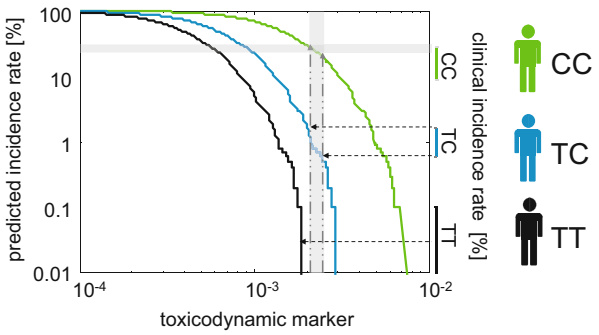

Fig. 10 Predicted incidence rates of ratios between mean muscle concentrations and simvastatin in vitro inhibitory potency (toxicodynamic marker) in populations of 1,000 virtual subjects for each genotype (*black line*, TT genotype; *blue line*, TC; *green line*, CC). Observed clinical 5-year incidence rates of myopathy (Link et al. 2008) are depicted as *vertical bars* to the right of the graph. Rates for TT and TC genotype subjects were used to determine thresholds for the toxicodynamic marker and to subsequently predict the higher risk of CC genotype subjects

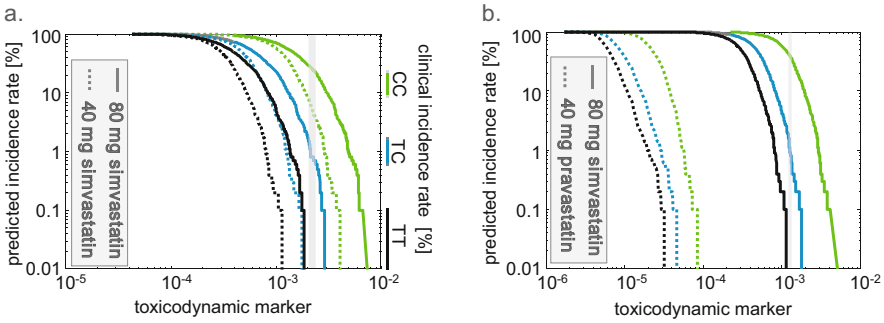

Fig. 11 Predicted incidence rates of ratios between mean muscle concentrations and simvastatin or pravastatin in vitro inhibitory potency (toxicodynamic marker) in populations of 1,000 virtual subjects for each genotype (*black line*, TT genotype; *blue line*, TC; *green line*, CC). *Gray bars* indicate thresholds derived from SEARCH data. (**a**) Comparison between 80 and 40 mg simvastatin. (**b**) Comparison between 80 mg simvastatin and 40 mg pravastatin

allows the identification of threshold toxicodynamic marker levels that can be used to predict the expected myopathy rate in subjects with the high-risk CC genotype. The model predicts incidence rates between 22.2% and 29.6% compared to 15.0% (CI: (8.8%; 23.1%) seen in SEARCH.

Moreover, the model and the identified toxicodynamic marker threshold can also be used to correctly predict the reduced incidence rates at lower simvastatin doses (Fig. 11a). Also the absence of myopathy events at pravastatin doses of 40 mg is predicted based on muscle concentrations (Fig. 11b) in contrast to the approach relying on blood concentrations. The model-based approach predicts an incidence rate of 0.04–0.11% for the low-dose simvastatin treatment compared to observed

0.097% in the SEARCH study. The predicted incidence rate for pravastatin is zero and no events were monitored in SEARCH.

The case study demonstrates how PBPK modeling and prior physiological and pharmacological knowledge may be used to predict clinically relevant events. Clinical incidence rates in dominant patient populations can be used to extrapolate to rare-frequency events in high-risk patients and how safety risks characterized for one drug and dose can be extrapolated to other doses and drugs from the same pharmacological class by use of prior information even in the absence a full-blown mechanistic understanding at all biological levels.

5 Conclusions

A good understanding of the causal chain from drug administration via systemic and tissue exposure to intended drug effects and adverse events is the determinant of success in clinical drug development. M&S helps to bridge gaps in our understanding and thereby reduces uncertainty and enables new quantitative insights. Today's modeling technologies allow very detailed integrated representations of biological, physiological, and pharmacological process at all levels from molecular interactions to organism-level physiology. Within the framework of detailed mechanistic modeling, knowledge gaps can be bridged using explicit assumptions or empirical relationships. State-of-the-art pharmacology uses M&S techniques to rigorously test hypotheses and plan new experiments.

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