Toxicodynamic Tests

17

Alexander Cartus and Dieter Schrenk

Contents

A. Cartus · D. Schrenk (✉)
Food Chemistry and Toxicology, University of Kaiserslautern, Kaiserslautern, Germany
e-mail: cartus@chemie.uni-kl.de; schrenk@rhrk.uni-kl.de

Abstract

Toxicodynamic testing is aimed at the elucidation of adverse effects of chemicals including understanding of their mode of action. In many cases, the "standard program" of toxicological testing on acute, subchronic, or chronic toxicity, genotoxicity, carcinogenicity, teratogenicity, and developmental and reproductive toxicity, which is needed for many regulatory purposes, already provides important information on the mode(s) of action of a compound. Targeted mechanistic investigations often follow, which use specifically designed models such as genetically modified cells or animals, studies using specific cell types, subcellular fractions, enzymes, etc. The understanding of the mechanisms underlying a certain mode of action and gained information on the dose- or concentration-response from in vivo or in vitro studies is crucial to derive point of departures for further human risk assessment and for regulatory toxicology of chemicals since it allows decisions on the options for extrapolation of experimental data to the human situation. This text follows the different levels of experimental models in toxicodynamic testing from isolated target molecules up to whole organisms like laboratory animals and humans.

Introduction

Depending on the special toxicological question addressed (Gregus and Klaassen 2001; Hayes 2001; Krewski et al. 2020), experimental models in toxicodynamic testing can make use of different hierarchical biological stages beginning with isolated target molecules like enzymes or receptors up to whole organisms like laboratory animals and humans as depicted in Fig. 1. Of course, biological complexity increases in this direction, possibly along with other factors like availability, price, or ethical issues or hindrances. In the following sections, we summarize and discuss options, advantages, and disadvantages of different experimental models in toxicodynamic testing.

Isolated Target Molecules

Nucleic Acids

Isolated nucleic acids of various degrees of purification can be obtained from different sources (DNA from calf thymus, herring sperm, tissue cultures, etc.) and be incubated with a chemical and/or its metabolites to detect if covalent binding occurs which may implicate a genotoxic/mutagenic mode of action. Reactive metabolites can be generated in situ by adding activating enzymes ("S9 mix") to the

Fig. 1 Levels of experimental models in toxicodynamic testing
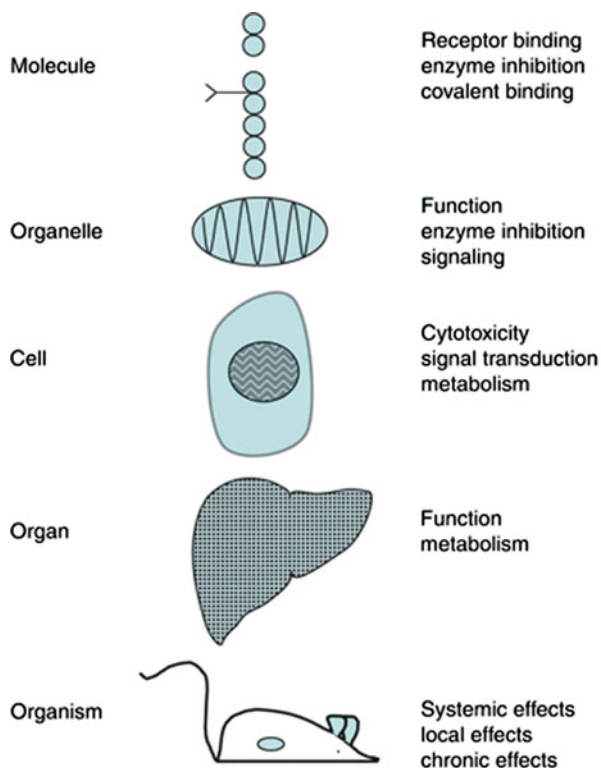
incubation. Subsequently, the nucleic acids are extracted, digested, and analyzed, e.g., for covalent binding of nucleosides to the chemical and/or its metabolites. Stable isotope-labeled DNA adduct standards can be added to the nucleoside preparation to quantify known DNA adducts most frequently via sensitive and specific LC-MS/MS methods. Alternatively, nucleotides (after hydrolysis) including modified nucleotides can be post-labeled with radioactive ^{32}P containing phosphate for further separation and identification via autoradiographic TLC (^{32}P-Postlabeling) which may help to screen for structurally unknown DNA adducts. Also, epigenetic alterations can be investigated in cell lines or in vivo, e.g., DNA methylation pattern or several histone (protein) modifications in intact DNA.

Proteins/Enzymes

The chemical or material of interest can be incubated with tissue or cell homogenates or with purified enzymes or other proteins. Assays are aimed at testing covalent or noncovalent binding but also functional effects on proteins (Pumford and Halmes 1997). Well-known examples are the inhibition of acetylcholine esterase by

organophosphates, binding of inhibitors of mitosis to tubulin in the spindle appara-
tus, or enzyme inhibition by certain heavy metals such as mercury ions. In the course
of such tests, information on the type of inhibition can be derived from concentra-
tion-effect analysis using a variety of inhibitor concentrations.

Lipids

Incubating purified lipids with test compounds or their metabolites can also be used
to identify possible covalent or noncovalent binding or, for example, to study lipid
peroxidation (via end products like malondialdehyde or 4-hydroxynonenal) as a
marker for oxidative stress in a cell or organism (e.g., TBARS (thiobarbituric acid
reactive substances) assay). Again, addition of an enzyme preparation can be used to
modify, e.g., activate, the test compound in purified preparations.

Subcellular Fractions/Organelles

Membranes: Cytoplasmic Fraction

The common method to isolate membrane fractions is sequential centrifugation. Like-
wise, a total membrane fraction can be isolated from a liver homogenate ultracentrifu-
gation at $100,000 \times g$, after nuclei, mitochondria, etc. have been sorted out at lower g
numbers. The supernatant of the membrane fraction represents the soluble cytosolic
fractions, sometimes called "cytosol." The sediment ("membrane fraction") can be
resuspended and subjected to additional (gradient) centrifugation in order to enrich
certain types of membranes. Following this approach, fractions enriched in endoplasmic
reticulum ("microsomes") – or outer cellular membrane-derived membranes – can be
prepared. The degree of enrichment can be verified by measuring the presence or
activity of marker proteins after addition of needed cofactors.

Such fractions can be used for the investigation of membrane-bound (CYPs,
UGTs, etc.) or cytoplasmic (GSTs, STs, etc.) enzyme activities, induction, inhibition,
etc. Furthermore, the metabolism of chemicals including genotoxic carcinogens,
leading eventually to mutagenicity, DNA binding, etc., can be analyzed. Together
with kinetic parameters obtained from such time- and/or concentration-dependent
experiments, also biokinetic properties and eventually DNA-binding activities in
vivo can be estimated (physiologically based biokinetic, PBBK modeling). The
supernatant of a $9000 \times g$ centrifugation of homogenized liver is called S9 mix or
S9 fraction, which contains microsomes and cytosol.

Receptors

In a strict sense, receptors act as triggers of signaling chains responding to agonistic
molecules by binding and change in receptor conformation. A typical consequence
of receptor activation is the formation of intracellular signal molecules called

"second messengers." Likewise, the binding of noradrenalin to β_1-adrenoceptors can result in enhanced intracellular formation of the second messenger cAMP. Xenobiotic chemicals can act on both membrane-bound receptors on the outer cellular membrane and on intracellular receptors, being located, e.g., in the cytoplasm or the nucleus. Also, trafficking of activated receptors, i.e., translocation from the site of ligand binding to the site of effect, is common. Xenobiotic ligands can mimic endogenous ligands, thus activating receptors thought to be responsive to hormones, transmitters, etc. In some cases, endogenous ligands are unknown ("orphan receptors"), or there is no scientific agreement on the identity of "the endogenous ligand" although a variety of endogenous compounds can bind to the receptor.

Effects of xenobiotic chemicals on receptors have been widely described and are considered as a central field in toxicodynamic research. In many instances, such effects are wanted, representing a fundamental mode of action of many therapeutic drugs. In toxicology, receptor activation can be crucial for many adverse effects. One example is the activation of hormonal receptors like ER (estrogen receptors), allowing to determine the "endocrine-disrupting" effect of chemicals in a direct receptor-binding assays or via reporter gene assays for chemicals that otherwise may not have toxicological adverse effects (like being genotoxic or carcinogenic, etc.). Also, the binding of dioxins to the aryl hydrocarbon receptor (AhR) is a prominent example. A major field of research on xenobiotic-responsive receptors is the adaptive response of drug-metabolizing enzymes called "induction of drug metabolism." This phenomenon, which can have adverse consequences for the organism, is used as a marker for certain types of receptor activation being monitored as a regulated battery of genes/enzymes. Some important examples for such concerted responses are given in Table 1 listing, e.g., the AhR, CAR (constitutive androstane receptor), PXR (pregnane X receptor; Fig. 2), or the PPARs (peroxisome proliferator-activated receptors).

Ligand binding to the receptor can be agonistic, partially agonistic, or antagonistic. This classification can depend on receptor subtype, cell type, species, etc. Furthermore, a compound can bind to an alternative ("allosteric") binding site on the receptor, thus modulating the affinity and/or effect transmission capacity of the "real" ligand which binds to the ligand binding site. These phenomena can be studied including binding assays in receptor-enriched tissue fractions or transfected cell lines which (over-)express the receptor of interest, e.g., combined with a specific reporter gene construct.

Transfer Through Biological Membranes (Ion Channels, Transporters, and Pumps)

In most cases, the function of ion channels, transmembrane transporters, and pumps is investigated using membrane fractions since most of these proteins are embedded in membranes. From the latter, vesicles can be prepared which can be used for transport studies, e.g., with radioactively labeled transport substrates. Such models are suitable for the analysis of the binding affinity of standard substrates, modulation of transport function, properties of a test compound as transport substrate,

Table 1 "Xenobiotic" receptors regulating expression of drug-metabolizing enzymes

Receptor	Chemical/compound	Inducible enzyme(s)
Aryl hydrocarbon receptor (AhR)	DL-PCBs, PAHs, TCDD	CYP1A1, 1A2, 1B1
Constitutive active (androstane) receptor (CAR)	DDT, NDL-PCBs, phenobarbital,	CYP2B1, 2B2, 2B6, UGT2
Peroxisome proliferator-activated receptors (PPARs)	Fibrates, phthalates (diethylhexyl phthalate)	CYP4A
Pregnane X receptor (PXR)	Clotrimazole, dexamethasone (rodents), HBCD, pregnenolone 16α-carbonitrile, rifampicin	CYP3A, 7A1, OATP2, MRP2, MDR1/Pgp
Nrf2 (via antioxidant-responsive element; ARE)	BHA, BHT, t-butylated hydroquinone	GSTYa, M, P1, NQO1

Abbreviations: *BHA* butylated hydroxyanisole, *BHT* butylated hydroxytoluene, *CYP* cytochrome P450, *DL-PCB* dioxin-like polychlorinated biphenyls, *GST* glutathione *S*-transferase, *HBCD* hexabromocyclododecane, *MRP* multidrug resistance-associated protein, *MDR/Pgp* multidrug resistance protein/P-glycoprotein, *NQO* NAD(P)H-quinone oxidoreductase, *NDL-PCB* non-dioxin-like polychlorinated biphenyls, *OATP* organic anion transporter, *PAH* polycyclic aromatic hydrocarbons, *TCDD* 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, *UGT, UDP*-glucuronosyl transferase
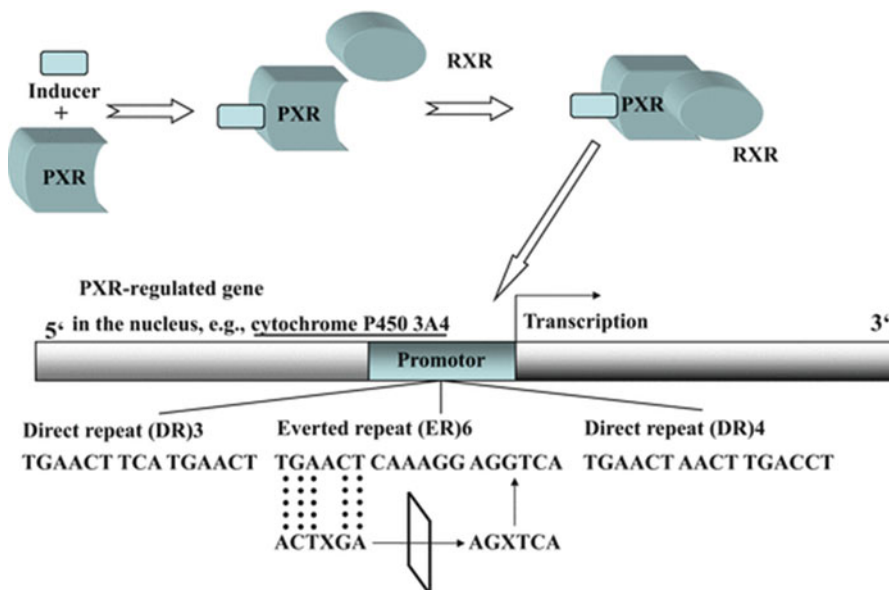

Fig. 2 Induction of gene expression via the pregnane X receptor (*PXR*). Upon ligand binding, the receptor dimerizes with the retinoid X receptor (*RXR*). The dimer binds to consensus sequences (direct repeats, inverted or everted repeats) in the 5′-flanking region of responsive genes, thus modulating their transcription

conformational changes in protein structure upon substrate binding, etc. Furthermore, cell cultures can be applied in order to investigate the consequences of a targeted overexpression of a certain transmembrane protein, its genetic elimination ("knockout"), or selective inhibition by antagonists.

Finally, transmembrane transfer proteins can be regulated at the level of gene expression and localization within the cell ("trafficking") or tissue, in tissue culture or whole organisms.

Mitochondria

Mechanistic studies in isolated mitochondria comprise the investigation of mitochondrial damage (loss of physiological function) and mitochondrial signaling. Mitochondrial enzymes involved in oxidative phosphorylation/ATP production and oxygen consumption ("respiratory chain") are typical targets of chemicals (blocking of respiration, uncoupling of oxygen consumption and ATP formation, etc.). Signaling compounds released by damaged mitochondria comprise cytochrome c, calcium ions, and many others. Gross change in mitochondrial function can be measured as changes in membrane potential, proton concentrations, oxygen consumption, calcium flow, ATP/ADP ratio, etc.

Nuclei

Isolated nuclei are used for mechanistic studies investigating effects of chemicals on gene transcription (nuclear run-on assays), covalent and/or noncovalent ("intercalation") binding to DNA, other types of DNA damage (e.g. by oxidation, strandbreaks), and modifications of chromatin and effects on nucleosomes or on DNA/chromatin processing enzymes (topoisomerases, nucleic acid polymerases, etc.).

Cells

Permanent Cell Lines

In contrast to many primary cells in culture, permanent cell lines always proliferate in culture being harvested from the culture plate and seeded onto empty cell culture dishes. This "passaging" can virtually be used as an infinite source of cells. However, permanent cells frequently change their properties after several rounds of passaging. Thus, the passage number should be provided as an additional source of information in experiments with permanent cells and tests with high passage numbers should be avoided.

Permanent cell lines are of limited use in the study of the mode of action of a chemical because they usually differ more or less from the corresponding primary

cell type. In many instances, permanent cell lines are derived from tumors exhibiting profound changes in genotype and phenotype when compared to normal cells. For the successful use of permanent cells lines, their properties should be investigated as far as possible. A focused analysis of effects on defined signaling pathways, which are known to be regulated in a similar way in primary cells, is a typical example for such use.

For instance, it has to be considered that ATP production in many classical proliferating cancer-derived cell lines is based on glycolysis under hypoxic conditions rather than oxidative phosphorylation which decreases cells susceptibility to mitochondrial toxicants. Thus, to study mitochondrial toxicity, glucose in the cell culture medium may be replaced by galactose to increase mitochondrial activity. Another important issue is to ensure that enzymes/transporters for the uptake of a chemical that has to be examined or enzymes to metabolize a chemical, e.g., to a mutagenic electrophile, are expressed and active in a used cell line. However, genetically modified permanent cells lines can be a well-suited tool to investigate several toxicological endpoints if it is warranted that all necessary enzymes are produced. Furthermore, genetically engineered permanent cell lines over- or under-expressing certain genes of interest provide a powerful tool to study the influence of the encoded proteins on various outcomes, pathways, etc.

Primary Cells and Organoids

Cells isolated from certain organs or tissues of humans or experimental animals such as liver, lung, kidney, or immune cells usually comprise a mixture of several cell types. The cell preparations are obtained, e.g., by perfusion of the organs with media which disintegrate the tissue or by lavage of the organ surface (e.g., pulmonary epithelia). Individual cell types, e.g., hepatocytes (liver), alveolar cells type I (lung), or macrophages (blood, tissues), can be prepared from mixtures of different cell types by sequential centrifugation/density gradient centrifugation. Many primary cell types can be seeded and adhere on uncovered or specifically covered cell culture dishes or tissue culture flasks. The culture conditions usually aim at keeping the cells as long as possible in their differentiated state, i.e., to maintain their tissue-specific ("in situ") properties and functions. In most instances, this aim cannot be achieved completely, and/or differentiation is partially lost during culture. Usually, permanent cells undergo senescence or lose their specific phenotype after a certain time in culture. This can partially be circumvented using 3D embedding or suspension techniques using extracellular matrix. Beside this, generating organoid structures from adult or pluripotent stem cells is a promising tool to study organ toxicity in a model near to the in vivo situation; however such models are not always commercially available (Messina et al. 2020). Parameters which allow conclusions on the mode of action of a chemical in cell cultures include cytotoxicity and cell death, effects on cell culture density, proliferation, apoptosis, as well as changes in protein synthesis or growth behavior (e.g., loss of contact inhibition, growth in soft agar). Likewise, the mechanisms leading to necrosis or apoptosis in cell culture are

investigated in detail (Wyllie 1997). Hallmarks of molecular pathways are activation of receptors (Fas receptor; TGF-ß1 receptor, etc.), mitochondrial signaling, changes in apoptosis-regulating factors (TNF alpha, bcl-2, bax, p53, etc.), or activation of caspases. In such investigations, various cell types equipped with different receptors as well as various derivatives of the test compound can be used. Furthermore, "omics" analyses detecting changes in gene expression (gene arrays, etc.), protein patterns (proteomics), and endogenous metabolites (metabonomics) play a more and more important role in identifying the cellular mode of action of a chemical but also need bioinformatic methods for their analysis due to the large amount of gained data. In more specific studies, secretion of certain growth factors or tissue hormones, matrix-cell interactions, release of transmitters, etc. are analyzed. The effects of such changes can be measured directly in co-cultures with respective responder cells (e.g., immune cells). In addition, certain biochemical effects such as enzyme inhibition, binding to nucleophilic targets, generation of reactive oxygen species, etc. can also be analyzed in primary cell cultures.

Of particular interest in toxicology is the investigation of genotoxic events in primary cells. These analyses comprise the determination of modified DNA bases, DNA fragmentation, mutations, micronuclei formation, chromosomal changes, DNA repair, etc.

Tissues

Isolated Organs

Isolated perfused organs such as the liver, lung, heart, intestine, or kidney from rat, rabbit, or guinea pig represent widely used models for the study of the mode of action of a chemical in toxicological research. They allow, e.g., the study of necrotic cell damage and its modulation by inhibitors of metabolic activation or by the addition of protective substances (e.g., of acetylcysteine in paracetamol-mediated liver damage). Furthermore, the issue of localization of the damage or of the underlying biochemical pathway can be addressed. Likewise, perfusion with an acute nephrotoxicant allows the determination of the exact site of tubular damage or the role of glutathione depletion in such a scenario. The perfusion rate (flow) and pressure characteristics can be of interest in analyzing the pathogenesis of a damage, e.g., in particular in lung or kidney. In addition, "functional" effects in an isolated organ such as changes in heart rate, uterus contraction, etc. can be detected. The duration of experiments with isolated organs is limited by the lifespan of the organ being between minutes and a few hours. In many cases, this time is sufficient, however, to obtain relevant amounts of metabolites from a chemical or sufficient organ damage, depending on the start concentration of substrate. A novel development in tissue research is the use of organs isolated from domestic animals such as pigs or cows from slaughterhouses. This method allows the reduction in numbers of experimental animals and benefits from the relatively close relationship between porcine and human physiology when compared to rodents.

Tissue Slices

Studies in tissue slices allow one to address many questions which can also be dealt with in isolated perfused organs or in cell culture. Thus, this model is positioned between cells and intact organs. Tissue slices are easy to prepare and use (no difficult preparation, no perfusion equipment, etc.) but lack the physiological perfusion via the blood vessels. Nevertheless, tissue slices in certain instances may allow relevant conclusions about the type of tissue damage, xenobiotic metabolism, and its modulation or complex changes in gene expression.

In Silico Methods

In silico methods are aimed to complement existing toxicity tests to predict toxicity, prioritize chemicals, guide toxicity tests, and/or minimize late-stage failures in drugs design. Those methods are interesting, because they are faster and cheaper than in vitro or in vivo experiments and of course for ethical reasons because no animal experiments are needed. As mentioned before, e.g., toxicokinetic parameters can be obtained by PBBK modeling using in vitro data. Also, for some toxicodynamic endpoints in silico methods are somewhat useful or are even already accepted in some regulatory fields. Methods include knowledge-based (i.e., decision trees have to be completed guided by rules defined by experts), QSAR models (quantitative structure-activity relationship; using a set of chemicals with known effect to span a domain in which the unknown chemical is inter- or extrapolated using different determinants) and read-across methods (based on structural similarities). The most developed endpoint in this regard is probably genotoxicity/mutagenicity, whereas other endpoints may be rather poorly predictable yet. For example, the risk assessment of genotoxic impurities in pharmaceuticals can be performed using different in silico methods (at least one knowledge-based and one QSAR model) and the TTC approach (threshold of toxicological concern) under EMAs ICH M7 guideline. Other endpoints/mode of actions that can currently be evaluated with different quality include DNA and protein reactivity, metabolism by cytochrome P450 and phase II enzymes, skin sensitization, or even carcinogenicity (genotoxic/non-genotoxic). Free and proprietary software tools are ToxTree, QSAR toolbox, Lhasa Nexus, Vega, and many others.

Adverse Outcome Pathways

An adverse outcome pathway (AOP) describes a series of so-called key events (KE) linked by key event relationships (KERs) on many hierarchical stages (from the molecular level to a whole organism) that are necessary to develop a toxicological adverse outcome, i.e., a disease or an effect like skin sensitization, followed by a molecular initial event (MIE). An important assumption of AOPs is that toxicological processes tend to share KEs and KERs, within an individual organism and also

across species. Furthermore, one MIE can be associated with different adverse outcomes and vice versa (Krewski et al. 2020). In case of skin sensitization, the MIE (after absorption) is the covalent reaction of a chemical with skin proteins, and KEs are keratinocyte response (activation of inflammatory cytokines), mobilization of dendritic cells and T-cell proliferation. All of these MIE or KEs are separately assessable with in vitro methods (see OECD Test guidelines: 428, 442C. 442D, 442E, 429). Together with the development of further specialized in vitro assays which address single KEs and MIEs as alternatives to animal testing and with in silico methods, assessment of AOPs may play a major role in the future in reducing and replacing animal experiments in line with the 3R concept.

Experimental Animals

Acute Toxicity/Organ Toxicity

Experimental animals represent the most relevant model for the comprehensive prediction of adverse effects of chemicals in humans. Also studies on the mode(s) of action of a chemical can be performed in animals covering many various aspects. For example, the effects of a chemical on certain enzyme activities, levels of hormones, growth factors, etc. in blood or target tissues can be investigated. Furthermore, a broad spectrum of parameters of organ function and morphology (histopathological analysis) can be carried out. From the complex picture thus obtained, conclusions can be drawn on the possible mode of action. These can be substantiated by the target application of modulators such as enzyme inhibitors. Furthermore, studies on effects on gene expression and transcription ("genomics/transcriptomics"), protein levels ("proteomics"), endogenous metabolites ("metabonomics"), or the metabolism of the xenobiotic chemical of interest ("metabolomics") are essential parts of the current broad approach in toxicological research.

Using modern methods of genetic engineering and breeding, genetically modified strains can be obtained which allows further conclusions on molecular targets. Examples are rodent strains with deleted or silenced genes ("knockout" animals) or strains which overexpress a certain homologous or heterologous ("humanized") gene. Likewise, the study of Ah receptor-knockout mice has provided crucial insight into the biology of this receptor and its role in dioxin toxicity. Another example is the use of DNA repair-deficient mice to investigate the role of DNA repair mechanisms on the genotoxicity of chemicals.

Chronic Toxicity/Organ Toxicity

The investigations (and prediction) of chronic adverse effects, i.e., lifetime exposure, e.g., over a period of 1–2 years for a chronic rat study with daily (or 5 days/week) treatment of a chemical via the appropriate route (oral, dermal, inhalation), represent

the most challenging task in toxicological research (see ▶ Chap. 9, "Examination of Acute and Repeated-Dose Toxicity"). The relevant changes are mostly unknown when the experiment starts. Furthermore, exposure in a certain time window may be the most relevant. In any case, animal experiments still are the most reliable tool in predicting chronic toxicity in humans. Crucial endpoints can be clinical, (histo) pathological, biochemical observations including weight gain, food/water consumption, organ weights, hematological changes, mortality rates, any morbidity and histopathological changes in organs, etc. For a more comprehensive overview, see OECD Test guideline 452 (Chronic Toxicity Studies). Accompanying in vitro studies can be applied to obtain more information on the molecular mechanisms or mode of action underlying adverse effects observed in chronic animal studies.

Other Modes of Action

Targeted analyses in animal testing are aimed at understanding mode(s) of action. They make use of the broad pattern of biochemical and pharmacological testing approaches such as changes in intestinal passage, blood flow, arterial blood pressure, bile flow, renal blood flow, and inulin clearance, to mention a few. However, a minor temporal change in bile flow or blood pressure does not necessarily represent an adverse effect since it also occurs under physiological conditions representing reversible, adaptive responses (see ▶ Chap. 43, "Adverse Effects Versus Non-adverse Effects in Toxicology"). Such observations can be very helpful, however, in the understanding of a mode of action and may even be useful in the development of new therapeutic drugs. Additional experiments frequently follow in order to clarify the molecular mechanisms leading to the observed mode of action, e.g., an induction of a biliary export pump in increased bile flow. The induction of drug-metabolizing enzymes is another example of a frequently observed, adaptive, and thus not necessarily adverse consequence of xenobiotic exposure in laboratory animals.

Genotoxic and Carcinogenic Effects

Mechanisms of genotoxic effects can be found in many of the aforementioned experimental models. Following the paradigm that mutagenic effects and primary carcinogenic ("initiating") lesions are permanent changes in nuclear DNA, the investigation of genotoxic events is focused on DNA. They include bacterial (Ames test, rec test) or yeast cells, mammalian cell lines (sister chromatid exchange, micronucleus test, HPRT assay, comet assay, etc.), or intact animals (mouse micronucleus assay) identifying DNA strand breaks, mutations, and aneugenic or clastogenic effects.

The enormous complexity of the carcinogenic process does not allow a comprehensive testing for carcinogenicity using short-term assays. Phenomenologically, carcinogenicity can be studied using laboratory animals (OECD Test guideline 451), most often rats and/or mice or non-rodent species. Although the main reason to conduct such a study is to obtain information on tumor formation and incidences in

different organs, data as mentioned for chronic studies (e.g., weight gain, hematological, clinical, biochemical data etc.) are collected as well. The multistage concept of carcinogenesis suggests the existence of a primary lesion, which predisposes the "initiated" cell for a development into a malignant cell passing various stages. These stages, also termed as promotion and progression, require the presence of additional factors which allows the cell to proceed on this way. It is unclear if these additional steps involve or even require specific genetic changes. Furthermore, predisposing genetic changes in "normal" cells may make those cells vulnerable to additional factors and may even be inherited by the organism. Examples for such predispositions are the familial polyposis coli with respect to colon cancer or the hereditary disposition for breast cancer. A widely used tool to investigate the multistage development of cancer is hepatocarcinogenesis in rodents. In this model, certain mutations in critical genes (hot spots), e.g., in the H-Ras proto-oncogene, are linked to the initiation step (Anderson et al. 1992). The subsequent phase of promotion can be facilitated by chemical factors (tumor promoters) which may inhibit apoptosis of initiated cells, e.g., by suppression of pro-apoptotic pathways or by inhibition of intercellular signaling, etc. Likewise, certain receptors, such as CAR (constitutive androstane receptor), PPAR alpha (peroxisome proliferator-activated receptor alpha), ER (estrogen receptors), and GHR (growth hormone receptor), can mediate the promotion effect. Detailed studies, e.g., with humanized mice have led to the suggestion that receptor-mediated liver tumor promotion, e.g., with phenobarbital, can markedly differ between rodents and human, depending on receptor-mediated signaling. These studies illustrate the difficulties in the use of rodent-derived tumor-promotion data in regulatory toxicology.

Teratogenicity and Developmental and Reproductive Toxicity

These investigations make use of almost all aforementioned experimental models using subcellular, cellular, organ, tissue, or whole animal systems. In addition to animal experiments in rodents, birds, and amphibians, mechanistic studies are aimed at the role of receptors (retinoid receptors, PPARs). Exposure of dams during pregnancy/lactation does ideally not lead to maternal toxicity. While malformations are frequently seen after birth, developmental effects can occur at later life stages or even only become visible at more advanced stages (learning behavior, etc.) or when the fertility of the offspring is investigated ("multi-generation study"). Detailed studies on reproductive toxicity of a chemical in experimental animals comprise macroscopic and microscopic investigation of changes in the reproductive organs, reproductive behavior, perturbations of steroid hormone homeostasis and metabolism, receptor-linked effects, etc. including an analysis of fertility and reproductive success. OECD guideline tests to study developmental and reproductive toxicity include the test guidelines 414 (Prenatal Developmental Toxicity Study), 416 (Two-Generation Reproduction Toxicity), 421 (Reproduction/Developmental Toxicity Screening Test), and 443 (EOGRTS: Extended One-Generation Reproductive Toxicity Study).

Alternatives to Animal Tests

In line with the 3R concept (replacement, reduction, refinement of animal testing) introduced by Russell and Burch already in 1959, many toxicity tests for certain endpoints/modes of action prior partly or solely performed using laboratory animals are nowadays replaced by in vitro and/or in silico methods as described above. Examples are, for example, the BCOP (Bovine Corneal Opacity and Permeability) assay for eye irritation or the 3 T3 NRU Phototoxicity Test. A complete, frequently updated status report on validated and accepted alternative methods can be obtained from EURL-EVCAM (2018).

Investigations in Humans

Toxicodynamic studies in humans include those during development of new drugs. Here, pharmacological studies can provide information on possible unwanted/adverse effects. Furthermore, interferences of chemicals with the signaling or metabolism of other compounds or substrates including endogenous compounds are of interest. In the field of receptors and drug-metabolizing enzymes, genetic polymorphisms have been identified in humans such as polymorphisms in the CYP2D6, CYP2C19, NAT2, GST μ, genes, etc. These can result in toxicokinetic effects on the fate of chemicals which may have strong implications for the toxicodynamics. The methods used to identify those polymorphisms comprise DNA investigations looking for individual point mutations or more frequent single nucleotide polymorphisms, as well as gene expression analysis such as RT-PCR, western blotting, enzyme assays, or next-generation sequencing methods. Metabolism tests in healthy human volunteers are widely used to investigate the consequences of genetic polymorphisms of this type on the kinetics of standard substrates such as caffeine (CYP1A2, NAT2), debrisoquine (CYP2D6), or chlorzoxazone (CYP2E1) (Keller et al. 2017).

Experimental studies on chemicals other than drugs have been carried out in human volunteers under strict ethical and technical rules aiming at the prevention of severe or sustained adverse health effects in the cohort. Epidemiological studies, both observational and interventional, can provide valuable additional information on possible correlations between exposure and adverse outcome in humans. These usually require, however, strong support from biochemical, cell culture and animal data to reach the level of causality.

Cross-References

▸ Adverse Effects Versus Non-adverse Effects in Toxicology
▸ Aims and Mission of Regulatory Toxicology
▸ Epidemiological Methods in Regulatory Toxicology
▸ Examination of Acute and Repeated-Dose Toxicity

▶ International Regulation of Toxicological Test Procedures
▶ Omics in Toxicology
▶ Specific Toxicity Tests for Neurotoxicity, Immunotoxicity, Allergy, Irritation, Reprotoxicity, and Carcinogenicity
▶ Toxicity Testing In Vitro: Regulatory Aspects

References

Anderson MW, Reynolds SH, You M, Maromnpot RM (1992) Role of proto-oncogene activation in carcinogenesis. Environ Health Perspect 98:13–24

EURL-ECVAM Status Report on the development, validation and regulatory acceptance of alternative methods and approaches (2018). https://ec.europa.eu/jrc/en/publication/eur-scientific-and-technical-research-reports/eurl-ecvam-status-report-development-validation-and-regulatory-acceptance-alternative-3

Gregus Z, Klaassen CD (2001) Mechanisms of toxicity. In: Klaassen CD (ed) Casarett and Doull's toxicology. McGraw-Hill, New York

Hayes AW (2001) Principles and methods of toxiocology, 4th edn. Taylor and Francis, New York

Keller GA, Gago MLF, Diez RA, Di Girolamo G (2017) In vivo phenotyping methods: cytochrome P450 probes with emphasis on the cocktail approach. Curr Pharm Des 23:2035–2049

Krewski D, Andersen ME, Tyshenko MG, Krishnan K, Hartung T, Boekelheide K, Wambaugh JF, Jones D, Whelan M, Thomas R, Yauk C, Barton-Maclaren T, Cote I (2020) Toxicity testing in the 21st century: progress in the past decade and future perspectives. Arch Toxicol 94(1):1–58

Messina A, Luce E, Hussein M, Dubart-Kupperschmitt A (2020) Pluripotent-stem-cell-derived hepatic cells: hepatocytes and organoids for liver therapy and regeneration. Cells 9(2):420

OECD Test guidelines. https://www.oecd.org/chemicalsafety/testing/oecdguidelinesforthetestingofchemicals.htm

Pumford NR, Halmes NC (1997) Protein targets of xenobiotic reactive intermediates. Annu Rev Pharmacol Toxicol 37:91–117

Wyllie AH (1997) Apoptosis: an overview. Br Med Bull 53:451–465