Chapter 35

Toxicity Methods

35.1 INTRODUCTION

As with other ADMET (absorption, distribution, metabolism, excretion, toxicity) properties, toxicity assessment uses a combination of in silico, in vitro, and in vivo tools to obtain data for key toxic mechanisms. A computational tool using topological polar surface area and $C\log P$ for toxicity is discussed in Chapter 17, Section 17.1. In silico tools have been developed for general compound trends that lead to toxicity and for predicting individual toxicity mechanisms based on the structure of a drug discovery lead. At the in vitro level, compounds are examined using assays for key indicators of toxicity. At the in vivo level, short- and long-term studies are performed for medically defined endpoints that are clear indicators of health. Toxicity is so critical to the success of developing a new drug that tremendous attention and priority are accorded to it. The predictability of each method for human clinical outcome is thoroughly examined. New in vitro methods should produce a minimum of false negatives, so that unexpected toxicities do not appear at a later stage. False positives are also problematic, because they place a stigma on the lead compound series. Indications of toxicity are a significant deterrent to further work on a compound or lead series, but can provide valuable information for structure modification. This chapter introduces drug discovery scientists to some of the terms and methods used in drug discovery and preclinical toxicity studies. It is not a detailed text and, for greater depth of understanding, detailed reviews, books, and experts should be consulted.

Toxicity studies during drug discovery either focus on key mechanisms of toxicity, or broadly search for signs of toxicity in living animals. Some toxicity results (e.g., genetic toxicity) can lead to termination of a particular drug candidate. There is a hierarchy of toxicity assays and resources applied to drug candidates:

- Earlier studies use higher-throughput in silico and in vitro methods to search for indications of the most common or critical toxicity mechanisms.
- Positive toxicity indications are followed up with sophisticated diagnostic testing.
- Advanced drug discovery leads or preclinical candidates are subjected to standard advanced procedures:
  - Short-term animal dosing studies
  - Broad off-target selectivity screening
  - In vitro studies of toxicity mechanisms that are not readily observed in short-term animal dosing studies

Most organizations [1] have toxicology groups to exclusively collaborate with drug discovery projects to allow the early identification and correction of toxicity liabilities. The advantages of early toxicity collaborations include the following:

- More informed decisions during drug discovery about lead prioritization
- Data that enable structure modifications of leads to eliminate toxicity during optimization stage
- Assure regulatory agencies that toxicity issues have been well investigated and do not pose a risk to human clinical volunteers
- Saving of resources from development on candidates that would fail owing to toxicity
- Optimum planning of human dosing studies in Phase I

The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) has published guidelines for regulatory safety testing of drug candidates. These cover carcinogenicity, genotoxicity, reproductive toxicity, immunotoxicology, and other toxicities. The details are found at http://www.ich.org/products/guidelines/safety/article/safety-guidelines.html.

35.2 IN SILICO TOXICITY METHODS

In vivo toxicity testing is expensive and requires a long time and a large amount of compound. Thus, in silico and in vitro tools have been developed for early testing. It has also been recognized that certain chemical moieties and structural
templates predictably induce certain types of toxic responses. This has lead to quantitative structure-activity relationship (QSAR) studies for the purpose of predicting toxic responses using in silico algorithms [2,3]. There are several limitations in the development of in silico models, including the quality and quantity of the toxicological data, the intricacy of each type of toxic mechanism, and the multiplicity of toxic mechanisms.

### 35.2.1 Knowledge-Based Expert System In Silico Methods for Toxicity

General classifications and expert opinions have been combined into knowledge-based methods. These provide rules for the evaluation of new chemical entities based on structural features, as well as toxicity probabilities. Thousands of compounds have been studied and thousands of rules have been developed.

Derek Nexus is an expert system offered by non-profit Lhasa Ltd. Rules about which substructures might be toxic are agreed on by a committee of experts and incorporated into the software. Predictions of toxicity, the rule(s) that indicated they might be toxic, and literature references justifying that a certain substructure might be toxic are shown on the screen, so the user can follow up. Meteor Nexus is a related product that predicts metabolites of a drug candidate, which can then be checked using Derek Nexus for potential toxicities. The Derek Nexus interface is reportedly easy to use and the system can be operated in batch mode. Derek Nexus predicts many types of toxicity. The U.S. Food and Drug Administration (FDA) has included Derek Nexus and Meteor Nexus in the process of reviewing new applications.

OncoLogic is a free downloadable software that uses a large array of rules. It was developed by the U.S. Environmental Protection Agency (EPA). The user interacts with the software to optimize the assessment. Carcinogenicity predictions and mechanisms are provided.

HazardExpert Pro evaluates compounds by toxicity associated with structural fragments. The software makes bioavailability and bioaccumulation estimations for inclusion in the toxicity predictions. It links to MetabolExpert to make predictions for compound metabolites that can be checked for possible toxicity.

### 35.2.2 Statistics-Based In Silico Methods for Toxicity

In these programs, parameters are calculated for structures and substructure connectivity. Computational models are derived using QSAR and neural net techniques.

Topkat uses a QSAR-based model. Structures are evaluated using electrotopological structural descriptors and fit with statistical linear regression and linear free energy relationships to produce the models.

Case Ultra divides the structure into moieties for pattern recognition and provides predictions for each. It interacts with Meta PC to predict metabolites for which toxicity predictions are made.

Software toxicity predictions are also included in the drug discovery ADMET products Percepta™ and ADMET Predictor™. Many companies have implemented one of these suites of ADMET predictors into their in-house desktop software for drug discovery scientists.

In silico toxicity prediction tools and other in vitro assay tools are listed in Table 35.1. Many large pharmaceutical companies develop in-house toxicity prediction tools based on their own data.

### 35.3 IN VITRO TOXICITY METHODS

As for other drug properties, in vitro assays are applied for toxicity screening and measurement [2]. This strategy allows drug discovery scientists to study a large number of compounds, examine specific toxicity mechanisms [3], use a low amount of compound, and reduce animal usage. In vitro methods are also applied at later stages to investigate specific toxicity questions, especially if they are not well covered by in vivo toxicity studies, such as mutagenicity, which leads to carcinogenicity. The following sections discuss many of the in vitro toxicity methods that discovery scientists use. The ICH provided guidance for many in vitro toxicity assays [4]. Ultimately, all in vitro assays are judged by their predictivity of in vivo toxicity results.

#### 35.3.1 Drug-Drug Interaction Methods

Co-administered drugs can interact to cause toxic effects, including cytochrome P450 (CYP) inhibition, metabolic enzyme, and transporter induction and transporter inhibition. The perpetrator drug changes the pharmacokinetics of the victim drug, which raises the victim drug exposure into its toxic range. CYP inhibition and transporter inhibition methods are discussed in Chapters 27 and 32, respectively.
### Table 35.1 Examples of Commercial Vendors of Products for Toxicity Methods

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<th>Topic</th>
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#### 35.3.1.1 Metabolic Enzyme Induction Methods

Some drugs induce the synthesis of certain drug-metabolizing enzymes and transporters (e.g., CYP, UDP-glucuronosyltransferases (UGT), P-glycoprotein). They initiate expression upregulation of the enzyme’s or transporter’s mRNA, which leads to synthesis and increased concentration of the enzyme or transporter. If a drug, which is primarily metabolized by the induced enzyme, is administered while the induced enzyme level is higher, metabolism will occur at a higher rate than normal. This can have two major effects, reduced efficacy and increased toxicity: (a) the unexpectedly high clearance of the victim drug leads to low exposure that reduces its therapeutic effect, and (b) induced metabolic enzymes increase the concentration of a reactive metabolite that causes toxicity (e.g., acetaminophen metabolized to reactive N-acetyl-p-benzoquinoneimine (NAPQI)).
To assess induction, the following three types of assays are used:

- Measurement of the metabolic enzyme mRNA
- Measurement of metabolic enzyme activity
- Activation of nuclear receptors (e.g., pregnane X receptor (PXR))

The FDA specifies the mRNA measurement method [5]. This method is described next, followed by the other methods.

35.3.1.1.1 Hepatocyte Method for Metabolic Enzyme Induction

Human hepatocytes are used for metabolic enzyme induction. Two induction endpoints can be measured: metabolic enzyme mRNA and enzyme activity. These two detection techniques for induction are discussed in the following sections.

35.3.1.1.1.1 In Vitro Measurement of mRNA for Metabolic Enzyme Induction

The standard assay for metabolic enzyme induction measures mRNA change in cultured human hepatocytes. Hepatocytes must be from ≥3 donors. CYP1A2, CYP2B6 and CYP3A are typically measured. In vivo studies can be followed to assess clinically relevant drug-drug interactions (DDI) [5,6]. Cryopreserved human hepatocytes from multiple donors are cultured in collagen-coated 24- or 48-well plates for 24 h. The test compound is added to each well on 3 consecutive days. The mRNA is then extracted and quantitated using real-time quantitative PCR (RT-PCR) TaqMan method.

From the concentration-response curves, $E_{\text{max}}$ and $EC_{50}$ can be calculated. A number of different approaches can be used to evaluate clinical DDI, including physiologically-based pharmacokinetic modeling (e.g., SIMCYP), static model, and relative induction score (RIS) method. The RIS is calculated using the following equation:

$$\text{RIS} = \frac{(C_u \times E_{\text{max}})}{(C_u + EC_{50})}$$

where $C_u$ is in vivo unbound plasma concentration, $E_{\text{max}}$ is the maximal effect, and $EC_{50}$ is the half-maximal effective concentration [7,8].

35.3.1.1.1.2 In Vitro Measurement of Enzyme Activity for CYP Induction

The higher induced levels of CYP isozymes by the test compound can be directly measured [9,10]. Hepatocytes are first exposed to the test compound for some time (e.g., 3 days). After time for induction to occur, the hepatocytes are incubated with substrates that are specific for each CYP enzyme of interest. The rate of metabolism by each CYP enzyme in the hepatocytes is measured, as in a metabolic stability assay. An increase in the metabolic rate for a substrate, compared to untreated hepatocytes, indicates that the CYP enzyme was induced by the test compound. The mRNA method described earlier was shown to be more sensitive and informative than the enzyme activity method [11].

35.3.1.1.2 In Vitro Nuclear Receptor Activation Methods for CYP Induction

The PXR regulates CYP3A, CYP2B6, CYP2C8/9/19 glutathione S-transferase, and other Phase II enzymes. The aryl hydrocarbon receptor (AhR) regulates CYPs involved in metabolism of aryl hydrocarbons (e.g., CYP1A2). The constitutive androstane receptor (CAR) regulates CYP2B6, CYP2C8/9, sulfotransferases, and glutathione S-transferases.

The mechanism of each receptor is similar. They detect the presence of xenobiotic compounds by their binding to a complex in the cytoplasm to which the PXR, AhR, or CAR receptor is bound. Ligand binding releases the receptor that moves into the nucleus, where it binds with retinoid X receptor (RXR) and together they bind to specific response elements of promoters that regulate the metabolic enzyme gene expression.

To measure PXR induction, a PXRE-luciferase reporter gene was engineered [12] in a special cell line. When the inducer activates PXR and binds to PXRE, luciferase is expressed and detected. Another PXR assay for high throughput is the competitive displacement time-resolved fluorescence resonance energy transfer (TR-FRET) assay, which is commercially available (see Table 35.1). It involves displacement of a proprietary FRET ligand by the test compound from a binding site on PXR.

An AhR assay [13] uses an AhR-mediated reporter gene expression (luciferase), called CALUX. A rat hepatoma cell line is stably transfected with the luciferase reporter gene, under control of dioxin-responsive enhancers. The cells are grown in 96-well plates for 24 h. Then they are treated with test compound. After 24 h, the medium is removed, the cells are washed, luciferase is extracted by lysis, and the luciferase activity is measured using a luminometer. Increased luciferase activity indicates increased AhR induction.

A CAR TR-FRET assay is commercially available (see Table 35.1). It involves displacement of a proprietary FRET ligand by the test compound from a binding site on CAR.
35.3.2 In Vitro hERG Blocking Methods

These assays test the blockage of the hERG K⁺ ion channel that can cause cardiac arrhythmia. hERG methods are discussed in Chapter 34.

35.3.3 In Vitro Genetic Toxicity Methods

Genetic toxicity often leads to carcinogenicity. It includes mutagens that change the DNA sequence by substitution, insertion, or deletion of base pairs. Several chemical mechanisms can cause mutations (e.g., reactive oxygen species and aryl epoxides form adducts with base pairs). Genetic toxicity also includes damage to chromosomes by strand breakage, termed clastogenicity. Thus, genetic toxicity is used as an early test of the carcinogenicity risk of a drug discovery compound. Often, a tiered strategy is used, where in silico tools are used to screen all compounds, higher-throughput assays are used for screening a smaller number of compounds indicated by the in silico tools, and an advanced or GLP level assay is run before full development.

Several in vitro and in vivo assays have been developed to detect DNA damage caused by test compounds. It has been shown that the use of two or more tests in parallel provides the highest sensitivity for detecting the mutagenicity of a compound [14]. These assays detect many carcinogens, but not all, especially those that work through other mechanisms than DNA damage. Many of the genetic toxicity assays first metabolically activate the test compound by adding liver S9 fraction and cofactors to the assay matrix, followed by a mutagen-detection procedure. This allows detection of whether the compound and/or metabolites cause DNA changes.

The ICH set guidelines for regulatory genetic toxicity testing. One option in the testing guidelines is: (1) in vitro test for gene mutation in bacteria (Ames test), (2) in vitro test for chromosomal damage, and (3) in vivo test for chromosomal damage in rodent hematopoietic cells. An IND includes the first two. Knowing these guidelines provides a view of what will be required of a drug candidate going to development, so that drug discovery scientists can avoid candidate termination.

35.3.3.1 In Vitro Ames Mutagenicity Method

The GLP Ames test is the gold standard assay for assessing the ability of a compound or its metabolite to cause DNA mutation [15,16]. Both frame shift and base pair substitutions are detected. Four mutant strains of Salmonella typhimurium and one mutant strain of Escherichia coli bacteria are used. Each bacterial strain used for the Ames test contains a base pair deletion that cause them to not be able to synthesize histidine (Salmonella strains) or tryptophan (E. coli strains) and only be able to grow when supplemental histidine or tryptophan are added to the growth medium. If a mutation occurs at the location of the histidine or tryptophan base pair deletions to make a functional enzyme, the subsequent bacterial generations can grow without the supplemental amino acids. The inclusion of liver S9 fraction in the assay enables screening of metabolites of the compound for their mutagenicity.

The bacteria are grown in culture medium overnight. Then, the test compound (e.g., 5 mg/plate), liver S9, and metabolic cofactors are added and incubated for 1 h. This solution is mixed with agar and plated (without the histidine or tryptophan). After 72 h of incubation, the number of colonies is counted. Greater numbers of colonies compared to control indicates a greater mutation rate. These colonies are called revertants.

The standard Ames test requires more material and time than commonly available during drug discovery, so material- and time-sparing assay variations have been developed and they have good agreement with the full Ames assay [15]. The “Mini Ames” and “Micro Ames” assays use fewer Salmonella strains and smaller scale (6 and 24 wells, respectively). The “bioluminescent miniscreen” uses transfected strains that produce a luciferase response when a mutation occurs, which can be detected with plate readers. “Ames II™” and “Ames MPF™” are liquid phase suspension assays that use colorimetric indicators to measure when bacteria have mutated to revertants. The “SOS Chromotest” uses a genetically modified E. coli strain that indicates when the SOS DNA repair damage system is activated (by use of a transfected lac Z reporter). The strain was engineered to lack the genes for DNA excision repair and to make the cell wall more permeable to mutagens [17–24]. One issue for higher-throughput Ames assays is that mutations occur rarely (1 in 10⁹ per cell per generation) [16], so validation to the GLP Ames test is important. These and other assays and their strengths and weaknesses have been reviewed in detail by a group representing major pharmaceutical companies [16].

Initial HT Ames assays are used to screen compounds. Full standard Ames assays are performed for drug candidates advancing to development to avoid attrition and provide information to go into the IND.
35.3.3.2 In Vitro TK Mouse Lymphoma Cell Mutagenicity Method

Like the Ames assay, the mouse lymphoma assay determines test compounds that cause gene mutations by base pair substitutions and frameshifts, but in mammalian cells. The detection system for this assay is mutation of the thymidine kinase (TK) gene to a nonfunctional form. TK’s normal function is phosphorylation of thymidine to produce thymidine monophosphate (TMP). The TMP concentration controls the rate of DNA synthesis in cells. A thymidine analog, trifluorothymidine (TFT), is introduced to cells after treatment with the test compound. Cells that have normal TK die, but cells that have TK mutations are unaffected by the TFT.

Mouse lymphoma cells, suspended in culture medium, are treated for 4 h with the test compound (over a range of concentrations), liver S9, and cofactors. Following this treatment, cells are centrifuged and washed to remove the test compound, resuspended, and incubated for 2 days. Cells are then seeded into 96-well plates containing TFT. After 14 days, the cell colonies are counted and the number of living colonies is indicative of the mutagenicity of the test compound [25–27].

35.3.3.3 In Vitro HPRT Chinese Hamster Ovary Cell Mutagenicity Method

This assay is another mammalian cell mutagenicity assay. The assay is sensitive to mutation of the HPRT gene to nonfunctional form. HPRT is involved in DNA synthesis. The detection system for this assay is that a nucleoside analog, 6-thioguanine (6-TG), is introduced to cells after treatment with the test compound. Cells that have normal HPRT die, but cells that have HPRT mutations are unaffected by the 6-TG.

Chinese hamster ovary (CHO) cells, suspended in culture medium, are treated for 4 h with the test compound (over a range of concentrations), liver S9, and cofactors. Following this treatment, cells are centrifuged and washed to remove the test compound, resuspended, and incubated for 3 days. Cells are then seeded into 96-well plates containing 6-TG. After 10 days, the cell colonies are counted and the number of living colonies is indicative of the mutagenicity of the test compound.

35.3.3.4 In Vitro Micronucleus Clastogenicity Method

This assay detects compounds that damage chromosomes or interfere with the cell division apparatus, to produce abnormal DNA fragments. During mitosis, the chromosomes do not migrate properly, because the centromere is damaged or lacking. The resulting broken DNA pieces or an entire free chromosome adheres to membranes and are called micronuclei, which can be observed by a microscope.

The assay in a single-point mode is conducted by incubating cells (e.g., CHO) in culture with test compound at high concentration (e.g., 10 mM), with and without metabolic activation. The treated cells are cultured after treatment. The cells are stained and examined by the microscope for the number of cells that contain micronuclei and how many micronuclei are present per cell. If the test compound has a dose dependency in producing micronuclei or if the production is reproducible, the test compound is classified as “positive” [28–30]. Advanced cellular image analysis and flow cytometry systems [15,31] are commercially available and have allowed more rapid unattended examination of micronuclei (Table 35.2).

35.3.3.5 In Vitro Comet Clastogenicity Method

Cells in culture (e.g., CHO) are treated with test compound, with and without metabolic activation, at high concentrations (e.g., up to 10 mM) for 6 h. The cells are then embedded in agarose on a microscope slide and lysed under mildly basic conditions. The lysed cells are then exposed to a mild alkali to unwind the DNA, allowing for the visualization of DNA damage and repair. The analysis of the comet tail length and DNA migration is used to assess the clastogenic potential of the test compound.

TABLE 35.2 Examples of Contract Research Organizations that Provide Toxicology Services

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<thead>
<tr>
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<th>Website</th>
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The cells are subjected to gel electrophoresis. DNA fragments from single- and double-stranded breaks and relaxed chromatin migrate toward the positive electrode, whereas unchanged DNA is too large to migrate past the nuclei. The fragments appear similar to a comet tail. The DNA is detected using fluorescence microscopy after staining. The relative fluorescence intensity in the tail (DNA fragments) indicates the frequency of DNA breaks. The method is also called single-cell gel electrophoresis assay. The method is very sensitive to DNA breakage caused by genotoxic compounds [32–34].

35.3.3.6 In Vitro GADD45a-GFP Mutagenicity and Clastogenicity Method

GADD45a (growth arrest and DNA damage) gene is a biomarker for genomic stress. It is involved with regulating DNA repair, mitosis delay, and apoptosis. It has been linked to green fluorescent protein (GFP) and transfected into human p53-competent TK6 cells [35]. This stable cell line (GenM-T01) is used in 96-well format for detection of genotoxins. Assay kits (GreenScreen GC and HC) are available from Gentronix. In initial tests, the assay performance exceeded other assays in sensitivity (success in detecting carcinogens) and selectivity (success in identifying noncarcinogens). In addition to mutagens, the assay detects aneugens (interfere with chromosome segregation in mitosis) that the micronucleus assay is normally used to detect. The presence of p53 in the cell line appears to be important in accurate induction of a cell’s response to genotoxins and is missing in cell lines used for some other in vitro genotoxicity assays. This high-throughput assay could precede current genotoxicity assays to increase efficiency and early detection.

35.3.4 In Vitro Cytotoxicity Methods

Cytotoxicity is a change in cell function induced by the test compound [36]. Many diverse mechanisms, by which the compound can impede the normal function of the cell, could potentially be assayed. Hepatocytes are advantageous for cytotoxicity assays because both the test compound and its metabolites can cause toxicity.

35.3.4.1 In Vitro ATP Depletion Cytotoxicity Method

Cells use ATP as their primary energy source. When cells become unhealthy, owing to many different toxicity mechanisms, the ATP usually is reduced. Standard assay kits are commercially available to measure ATP. Thus, ATP depletion is a general measurement for cytotoxicity.

One example of applying this concept uses THLE (transformed human liver epithelial) cell line that is available from ATCC. THLE cells in culture are treated at various concentrations of test compound for 72 h and then measured for ATP depletion. It was found that the odds of being toxic in an in vivo exploratory toxicity study at $C_{\text{max}} \geq 10 \mu M$, if the THLE LC$_{50} \leq 50 \mu M$, are fivefold higher than if the LC$_{50} > 50 \mu M$. Thus, the results from the THLE assay can be used to prioritize compounds for short-term (acute) in vivo toxicity studies (see below) [37].

35.3.4.2 In Vitro MTT Human Hepatocyte Cytotoxicity Method

This assay detects compounds that interfere with mitochondrial function. The indicator of healthy function is the reduction of yellow 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) to purple formazan by mitochondria. The concentration of formazan is measured by absorption at 570 nm.

Human hepatocytes or CHO cells are plated and cultured for 2 days. The test compound is added and incubated at concentrations of 10-1000 $\mu M$ for 24 h. The medium containing the test compound is removed and replaced with medium containing MTT. After 3 h incubation, the cells are lysed, formazan is extracted by organic solvent, and the absorption at 570 nm is measured. Absorption below that of the control indicates that the test compound is toxic to the cells.

35.3.4.3 In Vitro LDH Cytotoxicity Method

The plasma membrane of a dead cell will lyse and release the cell contents into the medium. Lactate dehydrogenase (LDH) is an abundant enzyme that is released. The concentration of LDH is measured using a colorimetric reaction. Cells in culture are treated with the test compound at a range of concentrations for 24 h. The medium is collected and tested for LDH activity with a test kit (Roche). Assays for other enzymes that are released by membrane lysis include alanine aminotransferase and aspartate aminotransferase.
35.3.4.4 In Vitro Neutral Red Cytotoxicity Method

Neutral red (NR) is a dye that is absorbed by hepatocytes and concentrated in lysosomes. It is a marker for healthy cells. Hepatocytes are treated with the test compound. Increasing uptake of NR is associated with increasing cell survival. This assay is claimed to be simpler and more sensitive than the LDH leakage test [38].

35.3.5 In Vitro Embryo Teratogenicity Methods

Embryonic developmental effects of drug candidates have been tested using a rat whole-embryo culture (WEC) model [39]. Rat embryos at 9 days development are cultured for 2 days, during early organogenesis, with test compounds at different concentrations. They are then microscopically examined for abnormal morphology development. This method has the highest agreement with the advanced in vivo rodent embryology models.

Zebra fish are used in a newer method [40,41]. They are small and easily maintained. They require only a small amount of test compound for dosing. Embryos are easily handled in 384-well plates. Their size and transparency enable them to be easily examined for specific developmental endpoints of teratogenicity studies. Abnormalities are readily observed. Embryos are treated with test compound for 4-6 h after fertilization and embryos are examined for 5 days for morphological abnormalities. The method successfully classified 87% of test compounds [41].

35.3.6 In Vitro Off-Target Selectivity Screens

To avoid disadvantageous off-target toxicity via activity at another target in the body (e.g., enzyme, ion channel, receptor), a screen of diverse targets is usually performed on selected advanced leads. This is usually performed by a contract laboratory (e.g., Eurofins Panlabs, Ambit Biosciences). A safety margin (see Chapter 17) versus these other targets is calculated.

35.3.7 In Vitro Reactive Metabolite Methods

Covalent binding of reactive metabolites to endogenous protein and its effects remains an area of research in drug discovery [42,43]. These assays examine the reactivity of compounds and metabolites with cellular components [44]. Metabolic reactions can produce reactive electrophilic intermediates or metabolites that covalently react with (e.g., protein) nucleophilic sites in the metabolic enzyme, liver cells, or another site in the body to cause loss of function and/or an immune response.

35.3.7.1 In Vitro Reactive Metabolite Method Using Glutathione Trapping

Glutathione (Figure 35.1a) is a ubiquitous peptide that traps reactive compounds (reaction of the thiol) and reduces oxidizers (reaction to glutathione dimer) to prevent damage to vital proteins and nucleic acids. Assays for reactive metabolites have been developed based on reaction of glutathione with reactive species. Glutathione is added to the in vitro metabolic stability method (see Chapter 29) that uses microsomes or hepatocytes to provide the metabolizing CYP enzymes. The thiol group of glutathione reacts at metabolically activated sites in the drug. The resulting glutathione and N-acetylcysteine adducts are extracted and analyzed using liquid chromatography-mass spectrometry (LC/MS/MS) techniques. Adducts are detected as their molecule ions or using a specific neutral loss (NL) MS/MS mode (e.g., NL of 129 in positive ion mode, NL of 272 in negative ion mode) [45–47]. This approach enables screening without needing to synthesize a radiolabeled drug candidate. The position of attachment of glutathione to the drug candidate can be elucidated using MS/MS and nuclear magnetic resonance (NMR) spectroscopy.

This method should be validated in-house and results should be confirmed using alternate methods, because it can produce false positives. Endogenous compounds can also cause false positives. Alternative approaches include the use of equimolar stable labeled glutathione analogs to produce a double-MS ion that is clear to see in the MS data, and the use of cyanide as a trapping agent. Advanced mass spectrometry scans and technologies (e.g., mass defect, data-dependent experiments, high-resolution MS) can be used to further accelerate the assay, provide fewer false positives, and quantitate the reaction products [48].

35.3.7.2 In Vitro Reactive Metabolite Method Using Covalent Protein Binding

A radiolabeled drug can be incubated (see Chapter 29) with microsomes or hepatocytes for 1 h. The protein is separated and analyzed for incorporated (covalently bound) radioactivity. A guideline for assessment is whether greater than 50 pmol equivalents/mg of the compound have been incorporated in the protein. The assay can also be performed in vivo [49,50].
35.4 IN VIVO TOXICITY METHODS

In vivo studies are of great importance because they are the ultimate indicator of toxicity in a complexly interacting living system. Standard short-term studies are required during the preclinical phase leading up IND and first-in-human (FIH) Phase I dosing.

35.4.1 Short-Term In Vivo Toxicity Methods

Acute toxicity studies are performed during drug discovery for selected lead compounds or as specific issues arise. A limited number of animals are used and studies are of short duration (e.g., 5-14 days). Many of the same parameters studied in preclinical toxicity studies are examined during drug discovery (e.g., safety pharmacology, postmortem histology), but in less detail and under non-GLP conditions. (Preclinical and clinical toxicity studies are discussed in the following section.) Discovery toxicity studies are sometimes conducted in coordination with in vivo pharmacology dosing studies, so that fewer resources are needed. For drug discovery lead compounds, the purposes of in vivo toxicity studies are the following:

- Check for any signs of toxicity
- Data for lead prioritization
- If toxicity is observed, follow up with toxicological examination of other leads

35.4.2 Preclinical and Clinical In Vivo Toxicity Methods

In vivo toxicity studies are described here in general terms to provide drug discovery scientists with an introduction to many of the tests that clinical candidates are subjected to once the drug discovery project team advances them to development. In vivo toxicity tests are required prior to FIH dosing and are performed under GLP conditions using highly detailed
A general scheme used in industry for preclinical studies, between candidate advancement and Phase I trials, is as follows:

- **Acute toxicity**: single dose
- **Chronic toxicity**: 2-14 weeks, daily dosing, rodent and nonrodent
- **Carcinogenicity**: 2 weeks, chronic dosing
- **Genotoxicity**: in vitro Ames test, in vivo mouse micronucleus test, chromosome aberration
- **Safety pharmacology**: monitoring of normal health, behavior, and function using medical examinations and tests for central nervous system, cardiovascular (including radiotelemetry), respiratory, gastrointestinal, and kidney. Tests include physical appearance, body weight, food consumption, eye function, ECG, blood chemistry, urine, and organ weight. Many of these studies are defined by the ICH guidelines [4].

A full microscope histology examination is performed on 50 or more tissues from the dosed animals.

Pharmacokinetic studies are often performed during the toxicity studies and correlated with toxicology studies. These are called toxicokinetics. Toxicokinetic studies allow determination of:

- no effect level (NOEL): highest dose or exposure that produces no toxicity
- no adverse effect level (NOAEL): highest dose or exposure that produces manageable toxicity
- therapeutic index (margin of safety): NOEL or NOAEL/efficacious dose or exposure

A candidate is terminated if severe toxicity is observed or if the therapeutic index is too narrow.

The purposes of preclinical studies are to predict patient hazards, insure a wide therapeutic index, plan clinical Phase I studies and dosing regimens, determine what organs are affected, determine toxicity markers to look for in humans at higher doses, determine any toxic metabolites, and examine drug responses that cannot be studied in humans [52].

More detailed animal toxicity tests are performed during the Phase I-III time period. These include:

- toxicity: 3-12 months, rodent and nonrodent (e.g., dog, monkey)
- reproductive health: mating behavior, estrous cycles, sperm, fertility
- embryonic development: survival, normal fetus and offspring growth, health and responses (rodent, nonrodent)
- oncology: 2 years, rat and mouse
- immunotoxicity (immunosuppression or enhancement) [53]
- toxicokinetics

### 35.4.3 In Vivo Toxic Biomarker Methods

In vivo toxicity studies have relied heavily on phenotypic response using histological examinations, the microscope examination of tissues prepared from dosed animals. New technologies have shown the ability to observe toxic responses at the biochemical level. These techniques profile small-molecule biochemical intermediates, proteins, and messenger RNA as biomarkers of toxicity. This approach can increase the number of compounds that can be evaluated and reduce the time required for toxicity studies.

#### 35.4.3.1 In Vivo Toxicometabonomic Method

It has been shown that toxicity can, in some cases, be detected earlier using spectroscopic analysis of body fluids [54–56]. The toxic activity, such as inhibition of an enzyme by the drug or a drug metabolite, will cause an imbalance in the normal biochemical intermediates in the organism. The concentrations of intermediates in the pathway of the inhibited enzyme increase or decrease. The study of endogenous biochemical intermediates is called “metabonomics” or “metabolomics.”

Animals are dosed once or daily for several weeks with the test compound. Urine and/or blood samples are collected and analyzed. The change in endogenous compound concentration is detected using LC/MS or NMR techniques. There are hundreds of components in these samples and sophisticated analysis methods are required. Detection of the affected endogenous compounds compares the spectra and chromatograms of samples from treated individuals to those of samples taken before treatment. One challenge is to determine if changes are due to normal biological fluctuations or due to effects of the test compound. In many cases biochemical changes are detectable in this manner before behavioral or morphological signs of toxic response are observed. The actual intermediates that change with dosing indicate which pathway is affected.
35.4.3.2 In Vivo Toxicoproteomic Method

In a similar manner to metabonomics, the balance of proteins can change in a biological system in response to the administration of a drug. Some of these changes are advantageous and consistent with the pharmacological goals for affecting the disease. Other protein pattern changes, however, are indicative of a toxic response. The study of the protein ensemble of a cell or an organism is called “proteomics” [57]. These studies use two-dimensional polyacrylamide gel electrophoresis (2D PAGE) and matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry analysis of protein mixtures from the sample.

35.4.3.3 In Vivo Toxicogenomic Method

Genomics is another indicator of toxic response. The mRNA in the cells is profiled to monitor gene expression in response to a toxic compound [58–61]. This technique is also called transcriptional profiling and the application of this technique has been termed toxicogenomics. cDNA and oligonucleotide microarrays are used to profile the thousands of mRNAs that might be modified by drug administration. Strong correlations have been reported between histopathology, clinical chemistry, and gene expression profiles [58].

PROBLEMS

1. On what are expert rule-based in silico tools for toxicity prediction based?
2. What are in silico toxicity tools useful for?
3. What is the gold standard method for CYP induction?
4. List some methods that can be used to detect cytotoxicity.
5. Compounds that damage DNA are which of the following: (a) enzyme inducers, (b) cytotoxic, (c) mutagenic, (d) potentially carcinogenic.
6. In the following table, link the assay with the observed endpoint:

<table>
<thead>
<tr>
<th>Assay</th>
<th>DNA Fragments Move Faster in Gel Electrophoresis than Normal DNA</th>
<th>Abnormally Divided Chromosome Is Observed as a Small Vessel by Microscope</th>
<th>Reversion Mutations Allow Colonies to Grow Without Histidine</th>
<th>Unusually Shaped Chromosomes</th>
<th>Normal Mammalian Cells Mutate so TMP Does not Kill them</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micronucleus</td>
<td></td>
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<tr>
<td>Comet</td>
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<tr>
<td>Ames</td>
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<tr>
<td>TK mouse</td>
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<tr>
<td>Lymphoma</td>
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</tbody>
</table>

7. The LDH assay works by which of the following mechanisms: (a) uptake by healthy cells is detected colorimetrically, (b) the LDH is taken up into the cells and reacts in the mitochondria to form a detectable product, and (c) lysis of unhealthy cells releases enzymes that are detected with a biochemical assay.
8. Teratogenicity can be determined using what?
9. What compounds are used to trap reactive metabolites?
10. What toxicity studies are always performed prior to human Phase I dosing?

REFERENCES


