Pharmacognosy Magazine
ISSN: 0973-1296

PHCOG MAG.
An official Publication of Phcog.Net

PHCOG MAG.: Recent Advances in Assay Methods and Techniques Preclinical Safety Studies on Medical Plants: Part II

Arun Kumar H.S.
Rudolf-Buchheim-Institute for Pharmacology,
Frankfurter Strasse. 107, D-35392
Giessen, Germany.
Correspondence: arun.kumar@phcog.net

Abstract: The use of various herbal remedies and preparations is increasing globally for various prophylactic and therapeutic purposes. As the interest in phyto-medicines and the use of plant derived extracts increases, so does the need to improve not only quality control methods but also authentication methods in terms of safety and efficacy. *"All substances are poisions; there is none which is not a poision. The right dose differentiates a poision from a remedy"* so said Paracelus many many years ago and today we adopt many approaches to identify this right dose range. Natural products have also fallen into this paradims of efficacy and safety assessment, although recently. Some of the approaches to safety assessment of medicinal plants are highlighted in this review.

Key Words: Medicinal plants, Phyto-medicines, Drug discovery, Toxicity studies.

Introduction

With the increasing use of herbal products as medicinal remedies, we are inundated with advertisements that tell us the natural remedies will accomplish cures and imply that the term "natural" means free of side effects (1,2). Many herbal products may do more than what they claim, and some of which may be undesirable. Unfortunately, only a few of the herbal remedies have scientific data to back up their safety concern. Some of the approaches to evaluate their safety and the recent advances in the field are described below.

Primary objective of toxicity evaluation studies

While the purpose of all toxicity evaluation studies is to assess the safety of the compound intended for clinical use by establishing acute, subacute/subchronic, chronic effects, potentiation with other phytochemicals, reproductive toxicity, teratogenicity, mutagenicity, carcinogenicity, effects on skin and eye, behavioural effects, immune effects and special toxicity profile, the following remain the primary objectives,

1. To identify the primary target organ(s).
2. To establish the reversibility of toxic lesion(s).
3. To determine the most sensitive diagnostic method(s) for detecting toxicity.
4. To indicate the toxic mechanism and probable antidote.

Steps involved in the safety screening process

I. Single dose toxicity tests

These studies are performed to define the target organ and evaluate the intrinsic toxicity of phytochemicals. They help in formulating safety measure during early stage of development and provide information for risk assesment of acute exposure and aid in designing and selection of dose for repeated dose toxicity studies.
In acute toxicity tests the compound is administered at different doses into two species (Rodent and non rodent) and by two routes of administration (one by intended route of use) to determine mortality/morbidity of dose. Fixed dose method/class method or up and down method is recommended for hazard classification.
Some of the non regulatory but equally specific tests specially concerning natural products safety evaluation are:

1. Brine shrimp lethality assay

The brine shrimp lethality assay is based on the ability to the extract to show lethality in laboratory-cultured *Artemia nauplii* brine shrimp. It is considered a useful tool for preliminary assessment of toxicity (3,4). It has also been suggested for screening pharmacological activities in plant extracts. The bioactivity of the plant extracts is evaluated with the shrimp lethality assay (lethality assay), as well as with another assay based on the inhibition of hatching of the cyst (hatchability assay).

2. Zebrafish toxicity assays

The Zebrafish has become a widely used model organism because of its fecundity, its morphological and physiological similarity to mammals, the existence of many genomic tools and the ease with which large, phenotype-based screens can be performed (5). Zebrafish promises to contribute to several aspects of the drug development process, including target identification, disease modeling, lead discovery and toxicology. Zebrafish screens for genetic or epigenetic perturbations that suppress a disease phenotype can be used to discover novel therapeutic targets. Large-scale, systematic screens can be performed in the zebra fish to identify small molecules that can suppress disease phenotypes. High-throughput Zebrafish toxicity assays combine many of the advantages of *in vitro* and *in vivo* toxicity models, making it possible to assess toxicity much earlier in the drug development process.

Acute organ specific toxicity tests are some of the recent developments in single dose toxicity assessment. Moreover they are high throughput based. Some of these tests are listed below:

1. Hepatotoxicity

These tests are performed to asses the hepatoxic potential and/or hepatic metabolism of the compound/natural product using either

1. Hepatoma cell culture,
2. Hepatic microsomal fraction
3. Hepatocyte culture
4. V79 chinese hamster cells.

2. Nephrotoxicity
The nephrotoxic potenential can be evaluated using the LLC – RK1 cell screening test or alpha methyl glucose uptake in cultured proximal tubular cells.

3. Cardiotoxicity
Assessment is done by embryonic myocardial myocyte reaggregates test and tissue excitable test using primary culture of myogenic satelites.

4. Haematotoxicity
A broad approach haematotoxicity test is performed by quantitating the Colony Forming Units in granulocyte/macrophage (CFU – GM) Assay.

5. Respiratory tract toxicity
Alveolar macrophage cultures can be used to assess the potential toxicity of the natural product extracts.

6. Single cell suspension/Isolated Tissue slice/Organ culture assay or methods
Although these are well in use for pharmacological studies, currently focus is on for extending their utility to toxicity screening as well. Liver slice, strips of arteries, ventricular papillary muscles, vascular beds segments, lung tissue and organ cultures such as Sub cellular fractions of liver, fibroblast, brain re-aggregate culture, renal proximal tubule, whole fetal heart, blood vessel, aortic rings and muscles co-cultures are some of which are routinely used (6,7).
Single cells from suspension or established cell lines of hepatocytes, neuroblastoma, progenitor and glial cells, cardiac and vascular-endothelial or smooth muscles, renal tubular cells, myocytes, embryonic tissue, myocardial cells and alveolar macrophage cells are also frequently used to assess single or repeat dose toxicities. Although the initial investments to such assays are very high, but in long run they prove to be quite economical.

II. Repeated dose toxicity studies
Groups of animals of either sex *(rats and dogs)* are treated with the extracts or phytochemicals via oral or parental route at different dose levels daily for 14/28/90 days; Satellite groups are included to check for the toxicokinetic parameters, reversibility and persistence of effect (2).
Study measurements include daily clinical observations, weekly body weights and feed consumption, ophthalmic examination, hematology, serum clinical chemistry, and urinalysis at pretest, interim and before termination. Complete necropsy is performed on any animal that dies or upon termination, and weights are recorded for major organs. Full histopathological examinations are conducted on all animals.

Immunotoxicological studies
Group of animal of either sex (mice or guinea pigs) are treated with test substance/ positive control for 30 days. Study measurements include clinical observations, body weights, gross necropsy and weights of spleen and thymus. Six animals from each group are immunized with keyhole limpet hemocyanin (KLH) at day 27 to induce antibody production and humoral immune response in immunized mice is measured by ELISA to detect anti-KLH IgM and IgG antibody titers. Hematology and spleenic lymphocyte immuno- phenotyping is also done in non-immunized mice at termination (8,9).
Cellular immune response in non-immunized mice is assessed by evaluating the T and B cell proliferation in response to stimulation with concanavalin A (Con A) and lipopolysaccharide (LPS) of gram negative bacteria. The ability of the extracts to trigger proliferation in an H-2-defined mixed lymphocyte reaction (MLR) on spleenocytes is also checked as an index of immunotoxicity.

Chronic toxicity/Carcinogenicity studies
Groups of animals of either sex (n=50) are treated with the test substance for 80/104 weeks. Satellite groups are also included for evaluation of non-neoplastic toxicity for 52 weeks. Study measurements include weekly clinical observations; body weights weekly for 13 weeks and monthly thereafter; weekly food and water consumption and ophthalmologic examination pretest and before study termination. Hematology, clinical chemistry, gross and histopathological examinations are conducted to assess the organ specific toxicity and analysis of neoplastic and non-neoplastic lesion (10).

Mutagenicity studies
Mutagenesis is an induction of alterations in DNA in either somatic cells or germ cells (11,12). These tests are of very much concern as somatic cell mutations may lead to cancer while germ cell mutations may lead to abnormalities in the offspring or foetal death. Hence mutagenicity tests must be peroformed with utmost care and often a battery of test are employed. A few of them are mentioned below.

1. Tests for assessing Gene Mutation
Salmonella reverse mutation assay (AMES test) (Prokaryotic)
The mutant salmonella are grown in media without histidine. Extract to be tested is added to growing bacteria. In the presence of a mutagens, the mutant Salmonella tends to mutate back to its original strain. So if there are any colonies growing after the test, then this indicates that a mutation has occurred. The Ames test employs several strains of *Salmonella typhimurium* which have been selected based on their sensitivity to mutation because of: 1) an increased cell wall permeability, due to mutation which causes partial loss of the lipopolysaccharide barrier that coats the bacterial surface and results in increased permeability to large molecules (rfa mutation); 2) a mutation in the bacterial cell system to excise and repair defects in the DNA, resulting in the inability to repair damaged or mutated sections (uvrB mutation); and 3) R-factor plasmids (some strains) and a multicopy plasmid (some strains) which contain error-prone DNA repair systems.
The test strains also require histidine for growth, due to a mutation in the gene, which controls production of histidine. The test strains employed are TA97A, TA98, TA100, TA102 and TA1535. The tests are performed both with and without S-9 activation. The S-9 activation system is designed to simulate mammalian liver enzyme systems and is used to detect substances, which undergo metabolic activation from non-mutagenic forms (13).
Mouse lymphoma assay/CHO-HGPRT assay (Eukaryotic)
These are mammalian mutation assay, which measures forward mutations at two different loci. The mouse lymphoma assay is an in vitro mammalian cell test capable of detecting compounds that induce either point mutations or other chromosome events. The cell line is heterozygous at the thymidine kinase (tk) locus.

Mutations or chromosomal damage involving the tk locus result in homozygous $tk^{-/-}$ cells with little or no tk activity remaining. When treated with trifluorothymidine (TFT), $tk^{+/-}$ cells incorporate the toxic analog into their DNA and die. Mutant $tk^{-/-}$ cells cannot incorporate TFT into their DNA, and therefore can grow to form colonies. The use of toxic nucleoside analogs forms the basis for cell selection following test article treatment in the mouse lymphoma assay.
Similarly the expression of hypoxanthine-guanine phosphoribosyl transferase (HGPRT) in Chinese hamster ovary cells is also assessed. In order to evaluate the effect of metabolism on the test extract, such tests are performed in the presence and absence of exogenous mammalian liver enzyme preparations (14).

2. Test for assessing chromosomal aberrations
a. Chinese hamster ovary (In vitro)
Cell cultures of Chinese hamster ovary cell line/ Human lymphocytes are expose to different concentration of test substance/ positive and solvent controls, with/without metabolic activation and harvest at 18h after exposure after colchicine administration.
Slides are stained using 5% Giemsa stain and 100/200 metaphases are observed from each group to calculate the mitotic index. Structural aberrations including chromosome and chromatids i.e. break, deletion, fragments and exchanges are record. Mice are also exposed to different concentration of test substance/ positive control and solvent (15).

b. Bone marrow cytogenetics/micronucleus assay
Five males and 5 females are sacrificed as per dose level per selected time point (6, 12, and/or 24 hr) after the exposure of test substance and bone marrow is collected after administration of colchicine to arrest cells in metaphase.
Slides are stained using May-Gruenwald solution and 5% Giemsa stain and 50 metaphase are observed from each group to calculate the mitotic index. Structural aberrations including chromosome and chromatid i.e. break, deletion, fragments and exchanges are record. Additionally polychromatic (PCE) and normochromatic (NCE) erythrocyte are also observed and ratio of PCE/NCE (cytotoxicity index) determined based on 200 RBC 1,000/2000 PCE per animal scored for micronuclei (11).

3. Tests to assay DNA repair
a. Rat hepatocyte unscheduled DNA synthesis (UDS) assay
These assays are designed to identify DNA repair synthesis after excision and removal of stretch of DNA containing a region of damage induced by test extract (16,17). The uptake of labeled nucleosides (tritium labeled thymidine) in cells that are not undergoing schedule (S phase) DNA synthesis is measured. These tests can be performed *in vitro* as well as *in vivo*.
In Vitro assays
Hepatocytes are exposed to ten different concentrations of test substance for different time points following which the cells are harvested and slides are stained using acridine orange. At least 30 cells are scored per slide and 3 slides per concentration are studied before any concurrent toxicity is evaluated.
In Vivo assays
Rat or mice of either sex are exposed to three different concentrations of test extract for 16-24 h, following which cells are harvested and stained using acridine orange. At least 90 cells are scored per slide and 3 animals per concentration of test extract are included.
b. Sister chromatid exchanges
Two rounds of chromosome replication are allowed to occur in the presence of BrUdr. Following which chromosomes are stained with Hoechst dye. The apparent exchange of segments of sister chromatids is called sister chromatid exchange (SCE) (18,19). The exchange of segments perpendicular to the chromosome axis implies that the chromosome is regularly compacted along the long axis of the chromosome. This compaction is consistent with the aggregation of the bases of loops onto a scaffold structure. Screening cell cultures for SCE's is a frequent method of testing chemicals for potential DNA damaging (and thus probably mutagenic) effects. SCE's are also elevated in genetic diseases of DNA repair.
Chinese hamster ovary cells are used for assessment of sister chromatid exchanges. Preliminary cell-cycle kinetics assay are performed with 5 concentrations of the test extract and definitive assay are performed latter with at least 3 concentrations of the test extract.
Assays are conducted with and without metabolic activation using duplicate cultures and at least 50 cells are scored per concentration to assess sister chromatid exchanges.
b. Differential killing of repair deficient and repair-proficient bacteria
Test for differential growth inhibition of repair proficient and repair deficient bacteria measure differences in chemically induced cell killing between wild-type strains with full repair capacity and mutant strains deficient in one or more of the enzymes which govern repair of damaged DNA (20). Bacterial DNA damage or repair tests measure DNA damage, which is expressed as differential cell killing or growth inhibition of repair deficient bacteria in a set of repair proficient and deficient strains. These tests do not measure mutagenic events per se. They are used as an indication of the interaction of a chemical with genetic material implying the potential for genotoxicity. Tests for differential growth inhibition of repair proficient and repair deficient bacteria measure differences in chemically induced cell killing between wild-type strains with full repair capacity and mutant strains deficient in one or more of the enzymes which govern repair of damaged DNA.

Primary dermal irritation study
The shaved intact skin of rabbit (n = 3-6) is exposed to the test substance for 4 hr. After the exposure period, the skin is graded for irritation at 1, 24, 48, and 72 hr. Observation period may be extended for up to 14 days to evaluate the reversibility of the effects observed.
Various new tests have been validated and regulatory agencies are reviewing the procedure and strategy for implementation:
Three *in vitro* assays, namely: EPISKIN™, EpiDerm™ (EPI-200), and the Rat Skin Transcutaneous Electrical Resistance (TER) Assay - have been evaluated as test methods for assessing dermal toxicity potential.
The EpiDerm™ (EPI-200) is a three-dimensional human skin model that uses cell viability as a measure of toxicity.
EPISKIN™ is a three-dimensional human skin model comprised of a reconstructed epidermis and a functional stratum corneum. The test material is topically applied

to the skin with subsequent assessment of the effects on cell viability.
In the TER Assay, test extracts dermal safety is identified by its ability to produce a loss of normal stratum corneum integrity and barrier function, which is measured as a reduction of the inherent transcutaneous electrical resistance below a predetermined threshold level.

Primary eye irritation study
Test substance is instilled into one eye of the rabbit (unwashed eyes), following which the cornea, iris, and conjunctival tissue of the treated eyes are graded for irritation effects at 1, 24, 48, and 72 hr (The redness is graded depending on the degree of redness and the distribution of the redness in the cornea, iris and conjunctiva). Observation period may be extended for up to 21 days to evaluate the reversibility of the effects observed (21,22).
A tired assessment procedure (using PH, dermal irritation data) is proposed.
Cell Culture (Frame Neutral Red Release/ cytotoxicity assay, Pollen tube growth test, Trans epithelial permeability test, etc.), Physiochemical (HET CAM test, EYETEX etc.), ex vivo (enucleated eye test using isolated eye) is under development.

Skin sensitization assessment
Preliminary primary irritation tests are performed to determine the maximum non-irritating dose. The skin of Guinea pigs is induced with 3 weekly 6-hr applications of the test substance. After a 2-week rest period, all animals are challenged with a single 6-hr application of a nonirritating concentration of the test substance. The skin is then graded for irritation at 24 and 48 hr after the challenge (21,22).
Murine Local Lymph Node Assay has replaced the above test by a few regulatory agencies.

III. Multi-generation studies
The aim of a multi-generation reproduction toxicity study is to detect any effect of the parent substance or its metabolites on mammalian reproduction (23). These include effects on male and female fertility, mating, conception, implantation, ability to maintain pregnancy to term, parturition, lactation, survival, growth and development of the offspring from birth through to weaning, sexual maturation and the subsequent reproductive function of the offspring as adults. While multi-generation studies are not specifically designed to detect developmental abnormalities because malformed offspring may be destroyed by the dams at birth, such studies may provide an indication of developmental toxicity if litter size at birth, birth weight or survival in the first few days after birth is reduced.
The study of more than one generation allows detection not only of any effects on adult reproduction, but also any effects on subsequent generations due to exposure in utero and early post-natal period. Critical aspects of development, which affect adult reproductive capacity, take place pre-natally and early post-natally. Adverse effects of sex hormones and their analogues administered during this critical period on reproductive tract development and function in males and females are well known. More recently, studies of other chemicals with endocrine disrupting potential have illustrated the critical role of exposure during the early developmental period on subsequent reproductive function in adult life. This can result in much greater effects on the reproductive capacity of subsequent generations compared with the original parental generation. Studies of more than one generation may also allow detection of reproductive effects due to bioaccumulation of the test substance. Interference with the developing reproductive tract or bioaccumulation may manifest itself via increasing degree.
Male rats are treated with test substance from 70 days prior to breeding, continuing up to matting period where as females are treated 14 days prior to breeding, throughout gestation and lactation period and till the termination of study. Study measurements include monitoring of estrus cycle, sperm morphology, sperm mortality, sperm count, pregnancy rate, litter size, litter weight, male: female ratio, male fertility index, female fertility index, live birth index, survival indices, gestation index, lactation index, neurobehavioral observation, body weight, food consumption and mortality rate. Gross and histopathological examinations are conducted to assess the organ specific toxicity.

Fertility and general reproduction performance study
Male rats are treated with test substance from 70 days prior to breeding, continuing up to matting period where as females are treated 14 days prior to breeding, throughout gestation and lactation period. Study measurements include clinical observations, body weight, food consumption, parental survival, confirmed mating, pregnancy rates, and length of gestation, number of live offspring, offspring survival, necropsy and histopathological findings. Bovine spermatozoa cytotoxicity test, in vitro fertilization sperm toxicity test and test using culture of human granulose luteal cells are developed and validation is underway (23).

Teratogenic tests
Pregnant females (rats/rabbits) are treated with the test substance at 3 dose levels daily during the organogenesis period. Day before parturition, animals are hysterectomized and sex, weight, number and location of viable fetuses, number and location of resorption sites, fetal weights and gross fetal abnormalities (External, Visceral and Skeleton) are determined. Additional study measurements include daily clinical observations and body weights daily during dosing period and on the day, when cesarean sections are performed (24,25). Embryonic stem cells, micromass, embryo culture method etc. are developed and validation is underway.

Prenatal and postnatal reproductive study
Groups of 30 female rats are treated with the test substance at 3 dose levels from Day 15 of gestation to lactation day 21. Study measurements include pregnancy rates, length of gestation, litter Observation (Number, sex, weights, still birth, live births, gross anomalies) clinical observations, body weight, food consumption and parental survival. Gross and histopathological examinations are conducted to asses the organ specific toxicity (24).

IV. Approaches to *in silico* analysis/*in silico* predictive toxicity
Toxicity issues are a significant factor affecting new drug candidate failures and R&D productivity. This has strengthened the importance of early screening for

toxicity properties. An emerging component of this screening strategy is the use of computer-based predictive models (*in silico*). Global and local (quantitative and qualitative) models are being applied to library design, to help prioritize synthetic programs, and to identify structural features which may contribute to undesired effects. In addition, searchable databases and database engines are marketed for data storage and retrieval. Predictive modeling, in conjunction with other screening technologies, has the potential to transform the drug discovery paradigm. However, to maximize the potential benefit from *in silico* technology it is critical that *in silico* models be applied judiciously and not over-hyped, moreover these can only be an additional support in the safety assessment process but not replacements.

The *in silico* approach can be either structure based or rule based:

1. Structure based

This approach involves identification of potential structural liabilities by collection of molecular fragments and descriptors, which is generally the database backup for all further assays. Values of chemical descriptors are calculated for all novel compounds and compared to known compounds based on which probability of being member of toxic class in a multifactorial statistical analysis is reported. Some of the currently available systems are:

a. TOPKAT

TOPKAT is a toxicity prediction program. TOPKAT uses Kier and Hall electrotopological states (E-states) as well as shape, symmetry, MW, and logP as descriptors to build statistically robust Quantitative Structure Toxicity Relationship (QSTR) models for over 18 endpoints (26). Another five models and endpoints are currently in development. TOPKAT runs on PCs under Windows 95/98/NT. TOPKAT can validate its assessments via a univariate analysis of the descriptors, a patented multivariate analysis of the fit of the query structure in Optimum Prediction Space, and by similarity searching in descriptor space.

TOPKAT can compute and validate assessments of the toxic and environmental effects of chemicals solely from their molecular structure. TOPKAT employs robust and cross-validated Quantitative Structure Toxicity Relationship (QSTR) models for assessing various measures of toxicity; the patented Optimal Predictive Space validation method assists in interpreting the results. TOPKAT can compute about 16 toxicity endpoints (i.e. Ames Mutagenicity, Rat Oral LD50, FDA Carcinogenicity, Developmental Toxicity, etc). TOKAT also allows users to perform similarity searching against the individual toxicity databases used to build and train the TOPKAT models. This database may be used for mining additional information about the toxicity of nearby neighboring structures or to find the journal reference information where the result was reported. However it is a closed system and doesn't provide any information on the metabolite prediction and its effects.

b. MULTICASE

Knowledge-based systems are computer programs aimed at organizing relevant experimental data for the purpose of helping a user make a decision about real world problems (27). CASE (Computer Automated Structure Evaluation) and its successor, MCASE/MC4PC, are designed for the specific purpose of organizing biological/toxicological data obtained from the evaluation of diverse chemicals. These programs can automatically identify molecular substructures that have a high probability of being relevant or responsible for the observed biological activity of a learning set comprised of a mix of active and inactive molecules of diverse composition. New, untested molecules can then be submitted to the program, and an expert prediction of the potential activity of the new molecule is obtained. MCASE differs from CASE in a great many ways, but the major algorithmic difference is the use of hierarchy in the selection of descriptors, leading to the concept of biophores and modulators. A new addition to these methods is the META program, an expert system capable, when coupled with appropriate dictionaries, to predict the metabolic transformations that may be produced when the molecules are ingested or dumped in the environment. The program is totally interfaced with MCASE and permits a complete evaluation of the potential toxicological effect of a molecule and its metabolites. The ability of these methods to predict activity in unknown molecules has been evaluated and compared to the results of equivalent experimental assays. The CASETOX program uses prediction modules developed by the MCASE/MC4PC programs and therefore can only use dictionaries provided by MultiCASE to effect its predictions. The CASETOX program is also useful to study toxic endpoints and has metabolite prediction option. However it is dated operating system and its different modules are not well integrated.

c. MISTA

MISTA (Multi-Intelligence System for Toxicogenomic Applications) is one such system designed to help drug discoverers avoid hundreds of billions of dollars in laboratory testing for novel compounds that ultimately fail, and reduce animal-based testing. It will close the gap between chemicals in use and chemicals with adequate toxicology data, dramatically reduce the time and cost required to bring new pharmaceuticals and chemicals into beneficial medical and commercial use, reduce the liability developers and commercial users face from currently unrecognized hazards, and reduce unanticipated human health and environmental impacts that result from deploying new chemicals with inadequate information. MISTA will screen drugs early during development, thereby reducing the enormous time and cost associated with failed drugs. MISTA bridges the understanding between physical and chemical properties and genomic/proteomic response by integrating three independent toxicity prediction tools operating in parallel. Each uses computational neural networks (CNNs) and wavelets to rapidly and accurately make pharmaceutical/chemical toxicity predictions. A CNN-based Quantitative Structure Activity Relationship (QSAR) module makes toxicological predictions based on chemical structure; a second CNN/wavelet module makes independent toxicogenomic predictions using micro array data; and a third CNN/wavelet module makes toxicogenomic predictions based on Massively Parallel Signature Sequencing (MPSS) data. This multi-intelligent, three-module approach provides crosschecks to reduce false positives and false negatives while substantially increasing confidence in predictions relative to current *in silico* toxicity prediction tools. MISTA is being developed with support from grants from the National Institutes of Health.

2. Rules based

This approach is based on inspecting molecules for known structural liabilities, based on which the program prepares a summary report of finding and thus identifies specific structural liabilities and gives references to the findings. Currently there is only one system available in this category:

a. DEREK

DEREK is a knowledge base expert system for the prediction of toxicological hazard (26). It is designed to assist chemists and toxicologists in predicting toxicological hazards based on an analysis of chemical structure. A session with *DEREK for Windows* is like holding a discussion with an expert toxicologist where reasoned opinions are sought rather than definitive answers. It uses a knowledge base, which contains alerts describing structure-toxicity relationships, with an emphasis on the understanding of mechanisms of toxicity and metabolism. Chemical structures can be easily inputted into *DEREK for Windows via* its automatic link to ISIS/Draw or by importing MDL Molfiles or SDfiles. During an interactive session, *DEREK* identifies the toxophore or substructure associated with toxicity and highlights this to the user with a brief statement about the hazard it represents. At the touch of a button the user can access additional information concerning the structure-toxicity relationship including literature references and supporting examples. The knowledge base covers a wide variety of important toxicological end points, which include carcinogenicity, mutagenicity, skin sensitization, teratogenicity, irritation, and respiratory sensitization. It is now well known that the physicochemical properties of a compound play an important role in determining potential toxicity. In recognition of this *DEREK* evaluates the predicted skin permeability of a chemical in order to predict its propensity to induce skin sensitization and photoallergenicity in humans. In the future, more of the physicochemical properties of a chemical will be considered in order to predict its potential toxicity over a wide range of end points more accurately. Further a user-friendly knowledge base editor is available that enables users to supplement the knowledge base with additional structure-activity relationships (SAR's), examples, and literature citations. Some of the disadvantages of the system include, difficulty in identifying new rules, metabolite prediction is not fully implemented, and it has rigid rule interpretation and includes no negative controls.

V. Emerging technologies

With the intention to reduce the animal use/suffering in toxicity screening process, several alternative methods are being developed. Brief overviews of all such approach are mentioned below.

1. Genomics

As genomics has revolutionized the study of biology, so has it affected how drugs are discovered (28). Pharmaceutical companies have also realized another major application: how drugs are assessed for safety. The analysis of gene expression profiles is now actively used alongside conventional toxicological assays to assess drug safety. Such toxicogenomic analysis is being used to predict drug toxicities and to gain a more in depth understanding of toxic mechanisms, so that more successful drug candidates can be selected. The use of toxicogenomics also has promise in proving hypotheses that support safe drug use in humans through a mechanistic understanding of toxicities found in drug-treated animals. A number of groups are actively addressing the issues of standardization of toxicogenomic data generation and exchange. The European Bioinformatics Institute (EBI) has created Tox-MIAMExpress to incorporate toxicity and toxicogenomics data into their Array Express database. Such efforts help identify the essential elements of a micro array experiment that are critical to interpreting a gene expression profile in the context of an associated toxicity. Some aspects of toxicogenomics experiments that are suitable for further standardization include data normalization methods, statistical assessments of gene expression, gene annotation and function, data visualization methods, and issues related to quality control of transcripts and probes.

The development of standards for toxicogenomic data generation and interpretation will help establish toxicogenomic approaches as scientifically accepted practices in the complex process of drug risk assessment. Central to this process is the continuation of the ongoing dialogue between molecular and traditional toxicologists from drug companies, regulatory bodies, and academia. The development of a scientific consensus on toxicogenomic data standards and data interpretation would mean fewer safety concerns and fewer delays in the drug approval process, resulting in improved availability of safe and effective drugs. The development and acceptance of toxicogenomic data submission standards promise to significantly improve the drug safety assessment process.

2. Transcriptome

It involves visualizing the expression level of genes on a chromosome or chromosomal region in a range of tissues and cell types following the exposure to the test extract. The user can select libraries of tissues and cell lines and compare their expression (29). Serial analysis gene expression (SAGE) libraries typically identify 50-100,000 mRNAs in a sample and the expression level of each gene is exactly quantified. These expression levels are related to all genes and algorithms are developed to identify transcriptional units (TU) in the human genome. The application has three levels. The 'whole chromosome view' (1) shows the expression level of all genes per chromosome. Clicking on a specific region leads to the 'concise view' (2), where details of the genes are given per chromosomal block of 25 genes. The 'extended view' (3) finally gives the details of the expression observed per SAGE tag.

3. Proteomics

The rapidly expanding field of proteomics relies heavily upon the use of two-dimensional gel electrophoresis (2-DE) or mass spectra of protein samples (30). 2-DE is a key separation technique in proteome analysis due to its advantage of simultaneous separation of thousands of proteins at a time, excellent reproducibility, and ability to exhibit post-translational modifications. Therefore, 2-D proteome analysis is becoming a popular method of choice to detect differentially expressed proteins between proteome profiles after exposure to toxicants. The proteomic investigation reveals a number of proteins that were up regulated or down regulated following the application of the test extract, most of the times in a dose-dependent manner. The advantage

of this approach is its ability to detect changes, which are not seen by reverse transcriptase-polymerase chain reaction. The determination of proteome changes following exposure to test extract will aid our understanding of the mechanisms of their toxicity as well as providing the possibility for the establishment of biomarkers that can be used in risk assessment as well as for the identification of individual susceptibility factors.

4. Metabonomics

Metabonomics is defined as the study of multiparameter responses of metabonome composition to various stimuli, including exogenous chemicals, pathophysiological conditions and genetic modifications (31). Metabonomics and the areas such as drug metabolism, metabolic fingerprinting, markers of disease and utilization of metabolites from animals, plants and microorganisms, require the development and use of highly efficient tools, including high-throughput technologies. Hyphenated methods and computerized databases allow rapid separation, quantification and structure elucidation in one working procedure, and provide the technological foundation for modern research and development related to low-molecular weight metabolites and metabonomics. It offers medium to high throughput, simple sample preparation process and aids identifying organ-specific changes and kinetics in animal or cell culture studies.

Although the emerging technologies seem to be of high utility by theoretical terms, how big their practical utility will be? Is something to be closely watched over in the coming years.

Concluding remarks

There has been a paradigm shift in the way the safety assessment has been approach in the past decade. As far as natural products are concerned most of safety assessment processes although similar to other conventional drugs have additional approaches to benefit from. Work towards evolving simple and effective approaches to improve the safety assessment processes are of great need as efficiency is directly linked to huge capitals. Most technique described in this text is constantly revised and updated to suite the need of the time and to meet the regulatory norms, hence it is very much necessary to keep updated.

References

1. Clardy, J. & Walsh, C. ***Nature*** 432, 829-37 (2004).
2. Arun, K. H. S. ***Pharmacognosy Magazine*** 1, 10-12 (2005).
3. D'Souza, P. et al. ***Phytother Res*** 16, 197-8 (2002).
4. Lee, S., Min, B. & Kho, Y. ***Arch Pharm Res*** 25, 652-4 (2002).
5. Zon., L. I. & Randall, T. P. ***Nature Reviews drug Discovery*** 4, 35-44 (2005).
6. Aleo, M. F. et al. ***Med Lav*** 93, 267-78 (2002).
7. Hildenbrand, S., et al. C. ***Arch Toxicol*** 74, 72-8 (2000).
8. Institoris, L. et al. ***Hum Exp Toxicol*** 20, 329-36 (2001).
9. Van Loveren, H. et al. ***Toxicology*** 185, 185-91 (2003).
10. Bolt, H. M. et al. ***Toxicol Lett*** 151, 29-41 (2004).
11. Jena, G. B. et al. ***Phytother Res*** 17, 306-310 (2003).
12. Jena, G. B. & Bhunya, S. P. ***Mutat Res*** 334, 167-174 (1995).
13. Lijinsky, W. et al. ***Nature*** 288, 589-90 (1980).
14. Wagner, V. O., et al. ***Toxicol Sci*** 55, 376-82 (2000).
15. Charles, J. M. et al. ***Mutat Res*** 472, 75-83 (2000).
16. Perez-Carreon, J. I. et al. ***Toxicol In Vitro*** 16, 253-8 (2002).
17. Le Hegarat, L. et al. ***Mutat Res*** 564, 139-47 (2004).
18. Siddique, Y. H. & Afzal, M. ***Toxicol Lett*** 153, 221-5 (2004).
19. Bhattacharya, K. et al. ***Toxicol Lett*** 153, 327-32 (2004).
20. Murialdo, H. ***Mol Gen Genet*** 213, 42-9 (1988).
21. Elmore, A. R. ***Int J Toxicol*** 22, 37-102 (2003).
22. Ballantyne, B. et al. ***Vet Hum Toxicol*** 39, 340-6 (1997).
23. Sullivan, F. M. ***Hum Toxicol*** 7, 423-7 (1988).
24. Deraniyagala, S. A. et al. ***J Ethnopharmacol*** 86, 21-6 (2003).
25. Birhanli, A. & Ozmen, M**.** ***Drug Chem Toxicol*** 28, 51-65 (2005).
26. Neal, F. C. et al. ***Mutagenesis*** 17, 321-329 (2002).
27. Rosenkranz, H. S. ***Altern Lab Anim*** 31, 393-9 (2003).
28. Lord, P. G. & Papoian, T**.** ***Science*** 306, 575 (2004).
29. Caron, H. et al. ***Science*** 291, 1289-1292 (2001).
30. Aebersold, R. & Mann, M. ***Nature*** 422, 198-207 (2003).
31. Nicholson, J. K. et al. ***Nature Reviews Drug Discovery*** 1, 153-161 (2002).