M.Pharm-Pharmacology
Pharmacological and toxicological screening Methods-II
(MPL202 T)

Course teacher: Dr.V.Parthasarathy-Professor, Department of Pharmacy

Chapter3. Reproductive Toxicology Studies

1. Reproductive toxicology is nothing but the harmful effects exerted by drugs and chemicals on the progeny and/or impairment of male and female reproductive functions leading to infertility as well as teratogenicity.

2. The reproduction and neurodevelopment are complex, stepwise processes, which starts with gametogenesis, followed by gamete interactions, implantation, embryonic development, growth, Parturition, post natal adoption.

3. Testing of developmental and reproductive toxicity in nonhuman primates (NHPs) is becoming more common with biopharmaceuticals in drug discovery and development.

4. Hence, the reproductive toxicology studies should be carried out in two species namely, rats and rabbits and it must be completed prior to phase trials.

5. The developmental and reproductive toxicity must be carried out with NHPs such as rodents and rabbits since, this species only express pharmacologic responses similar to humans. But it has the limitation that the human and animals are differing in toxic kinetic studies. Hence, the data on toxicity from animal must be carefully interpreted and must be reviewed carefully.

6. The toxicity of the chemicals/drugs must be mediated through either activation or detoxification of biotransformation pathways. Hence, it is essential to establish whether the routes and rates of human and animal metabolic pathways are similar.

7. The reproductive toxicity studies must be carried out as per ICHS5 Guideline and it was adopted in 1993.

8. The developmental toxicity such as (a) embryo-fetal development; (b) pre-postnatal development and (c) enhanced pre-postnatal development); (d) reproductive toxicity studies in male and female as well as juvenile toxicity study are carried out in non-human primates.

9. Various phases of reproductive toxicology are as follows.

10. **Developmental toxicity:** An adverse effect induced prior to attainment of adult life, which includes the effects induced or manifested during embryonic or fetal period and manifested postnatally.

11. **Embryo/fetotoxicity:** It is nothing but any toxic effect resulting from prenatal exposure, which includes structural and functional abnormalities as well as postnatal manifestations of such effects.

12. **Teratogenicity:** It is a result of developmental toxicity, which represents as a result of embryo/fetotoxicity due to the induction or the increase of the frequency of structural disorders in the progeny.

13. This study should be performed to examine whether the drug is teratogenic or has an effect on perinatal/postnatal development. Studies designed to assess the teratogenic potential should be carried out in two species, usually in rats and rabbits.

14. In the early 1960s administration of the mild sedative thalidomide, resulted characteristic reduction deformities of the limbs of fetus and it is ranging from hypoplasia of one or more digits to the total absence of all limbs.

Male reproductive toxicity study

Markers for physiological damage concerning to male reproductive system.

(a) In vitro sperm analysis, which measures the rate and extent of the animal and human sperm fertilize oocytes.

(b) Sperm function assays.

(c) Computer based automatic analysis to characterize the spermatozoa such as counts, motility, structure, domains and enzyme function.

(d) Specific markers and cDNA probes to testes and individual accessory sex organs.

(e) Molecular probes for RNA, DNA and other macromolecules to quantify specific steps in the development of germ cells.

(f) Analysis with these markers exhibit epididymal function, particularly sperm motility and its fertilizing ability.

(g) Assay of inhibin, androgen-binding protein as well as Mullerian-inhibiting factor and other polypeptides involved in fertilization.

(h) Enumeration of spermatogenic stem cells etc.

Female reproductive toxicity study- segment I, II and III

Various segments in reproduction toxicity are classified as: Segment 1 (Fertility), Segment 2 (Embryotoxicity / Tetragenicity and Segment 3 (Pre/post natal toxicity).

Various processes of segments are as follows:

Segment 1: Productions and release of gametes, fertilization, transport of zygotes and implantation.

Segment 2: Embryogenesis, Fetal development.

Segment 3: Parturition, lactation and postnatal development, Development and growth.

Various drugs and chemicals causes reproductive toxicity are as follows.

a. Abnormal foetal growth and development caused by Alcohol.
b. Abnormal foetal neurobehavioural development caused by heroin, cocaine.
c. Foetal malformations at any stages of the cycle caused by Thalidomide (anti-emetic), Gossypol (antimalarial), DES (synthetic oestrogen), Ergotomine (anti migraine drug) and aspirin (acute pain).

Genotoxicit study:

Genotoxicity is described as a property of chemical agents that damages the genetic information within a cell and cause mutations. The g**enotoxicity** cause cancer, neuronal disorders etc. All mutagens are genotoxic in nature.

The genotoxin cause DNA damage in cells when exposed and the DNA damage can be in the form of single and double strand breaks of DNA, loss of excision repair, cross-linking, point mutations, structural and numerical chromosomal aberrations, which may lead to cancer and other genetic disorders.

Many sophisticated techniques including Ames Assay, *in vitro* and *in vivo* Toxicology Tests, and Comet Assay have been developed to assess genotoxic potential of chemicals to cause DNA damage that may lead to cancer.

References:

1. Birth Defects Res (Part B) 86:446–462, 2009.2009 Wiley-Liss, Inc.
2. Guideline for Industry, Detection of Toxicity to Reproduction for Medicinal Products.' Center for Drug Evaluation and Research, 5600 Fishers Lane, Rockville, MD 20857.
3. General commentary on drug therapy and drug risks in pregnancy. Paul Peters, ... Christof Schaefer, in Drugs During Pregnancy and Lactation (Third Edition), 2015.
4. J. Gelineau-van Waes, in Comprehensive Toxicology, 2010.

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Unit: 5 Toxico-kinetics

Toxico-kinetics is defined as the generation of pharmacokinetic data to design, conduct and interpretation of drug safety evaluation studies. This study reports the adverse effects and mechanism of action of a chemical substance/drug molecule.

The toxico-kinetic study involves preclinical drug safety evaluation and toxicological studies. To carry out the toxic kinetic studies minimum of two animal species such as (a) Rodents such as Rats and Mice; (b) Non-rodents like dog will be employed as per FDA regulation.

Toxicokinetic studies in preclinical study are namely (a) Safety assessment; (b) Single dose and rising dose studies:

(a) Safety assessment:

It is an integral part of the study. The safety of a molecule can be performed in *in-vivo* system to assess the systemic exposure.

(b) Single dos and rising dose studies:

This study usually performed in rodents. Toxicokinetics study investigate how disposition kinetics of exogenous drugs/chemicals derived from natural or environmental sources generally referred to as xenobiotics show their deleterious effects on animals and including humans.

The plasma or serum samples of animal after exposure to drug molecule will be tested its ADME properties. This study help the researchers to chose the mode of administration of drug and the choice of formulation for better therapeutic efficacy. The results of single-dose kinetic studies will help to predict rate and duration of exposure during a dosage interval. The rising –dose studies performed in non-rodent models useful in evaluating the toxicokinetic study at various time points for each new dose level.

Toxicokinetics studies generally include repeated-dose toxicity studies, reproductive, and carcinogenicity studies. Since, single dose studies are usually done before a bioanalytical method has been developed, toxicokinetic monitoring cannot be integrated in these studies. Data on systemic exposure becomes particularly important in cases of negative results of *in vivo* genotoxicity studies.

The uncertain factors associated with toxicological risk assessment may be reduced Important factors include route of exposure, exposure pattern, exposure duration, physical exercise, body build, enzyme induction, enzyme inhibition, and species differences in size, physiology, and metabolism.

Applications of toxicokinetic study:

The main purpose of toxicokinetics is to describe the systemic exposure achieved in animals and its relationship to dose level and the time course of the toxicity study. The data relates the exposure achieved in toxicity studies useful in the assessment of the relevance of these findings to human safety.

Toxicokinetic data helps to determine the appropriate species, study design, and treatment regimen in subsequent non-clinical toxicity studies. Toxicokinetic study provides information on linear/non-linear pharmacokinetics, accumulation, and

whether the effects are related to C_{max} (peak concentration) or total exposure (AUC).

The modeling approach of toxicokinetics relates to the target dose concept, that is that the toxic effect of a chemical only arises when the chemical or its metabolite (s) reach specific targets within the body and a quantitative relationship between target dose and toxic effect can be expected.

Toxicokinetic information also helps in evaluating the impact of a proposed change in the clinical route of administration . Normally, in the case of large animals, blood samples for the generation of toxicokinetic data are collected from main study itself. However, in the case of smaller species satellite groups may be required.

Toxicokinetic models may also be used to strengthen experimental results by linking data obtained under different experimental conditions in a uniform model.

Method

Toxicokinetic study is an important tool to relate the dose of a drug or the concentration of a chemical with mode of action of the chemicals and its various metabolites. The toxicokinetic process is playing a pivotal role in the distribution and formation of various chemical entities at the target tissue, which is further responsible for determining the dose at the toxicological site.

The rate and magnitude of absorption, distribution, metabolism, and excretion process determines the dose/concentration at the target tissue. The basic toxicokinetic parameter is based on in vitro and in silico analysis using computational biology, which detects the potential of accumulation as well as potential of distribution or inhibition of chemicals in the tissues/organs.

Toxicokinetic models can be divided into two broad categories depending on the function of time and dose: data-based compartmental models and physiologically based compartmental models.

In vitro approaches retrieve information of prime importance in the area of toxicokinetic studies. One of the representative model, the physiologically based toxicokinetic model, can be obtained by including the kinetics of metabolism by the liver and any other organ capable of biotransforming the compound like lung, tissue–blood partition coefficients, and involving the transport process kinetics.

Acute systemic toxicity of any chemical can be easily predicted by incorporating the basal cytotoxicity data along with the results revealed by toxicokinetic models, which purely depends on the concentration and time course in separate tissues.
ICH Guidance S3A provides detailed recommendations on toxicokinetic assessment.

Reference:

1. Debra Kirchner, Susan Henwood. The Laboratory Rabbit, Guinea Pig, Hamster, and Other Rodents, 2012
2. P.K. Gupta, in Fundamentals of Toxicology, 2016
3. Abhishek K. Jain, ... Alok K. Pandey, in In Vitro Toxicology, 2018
4. Gursharan Singh, in Pharmaceutical Medicine and Translational Clinical Research, 2018.

Alternative methods in animal toxicity testing

(A) Prediction of toxicity of a drug molecule/chemical using in silico analysis

Assessing the toxicity of a drug/chemical is essential to identify it's harmful effects on humans, animals, plants, or the environment. It is a one of the important steps in drug design. Animal models have been used for a long time for toxicity testing. However, *in vivo* animal study is associated with limitations such as time, ethical considerations, and financial burden. Therefore, computational methods for testing the toxicity of drugs and chemicals are considered useful.

In silico toxicology is one type of toxicity assessment technique uses computational methods to analyze, simulate, visualize, or predict the toxicity of a drug and chemicals. In silico toxicology to complement to existing toxicity tests and it to predict toxicity, prioritize chemicals, guide toxicity tests, and minimize late-stage failure in drug discovery.

There are various methods for generating models to predict toxicity such as any undesirable or adverse effect of chemicals. Specific types of these adverse effects are called toxicity endpoints, such as carcinogenicity or genotoxicity, dermal toxicity etc. In Silico toxicity study predicts either quantitative e.g., LD50 (lethal dose to 50% of tested individuals) or qualitative, such as binary e.g., toxic or non-toxic or ordinary e.g., low, moderate, or high toxicity.

I*n vitro* toxicity tests became plausible due to the advances in high throughput screening such as i*n silico* toxicology (computational toxicology) is a type of toxicity assessment that uses computational resources such as methods, algorithms, software, data, etc. to organize, analyze, model, simulate, visualize, or predict toxicity of chemicals. I*n silico* pharmacology uses information from

computational tools to analyze beneficial or adverse effects of drugs for therapeutic purpose.

The molecular modeling software suite" Schrödinger and Discovery studio are helping the scientist to screen the drug molecule and to predict its toxicity. The various modules of Schrödinger Maestro are as follows: (a) The Bioluminate is an excellent tool in protein-protein docking, protein modeling, protein sequence analysis and prediction of toxicity (Tox) as well as ADME properties analysis.

Advantages of in silico analysis for toxicity prediction

Computational methods aim to complement *in vitro* and *in vivo* toxicity tests to potentially minimize the need for animal testing, reduce the cost, time of toxicity tests, and improve toxicity prediction and safety assessment.

In addition, computational methods have a unique advantage of being able to estimate chemicals for toxicity even before they are synthesized.

In silico toxicology encompasses a wide variety of computational tools such as: (A) databases for storing data about chemicals, their toxicity, and chemical properties; (B) software for generating molecular descriptors; (C) simulation tools for systems biology and molecular dynamics; (D) modeling methods for toxicity prediction; (E) modeling tools such as statistical packages and software for generating prediction models; (F) expert systems that include pre-built models in web servers or standalone applications for predicting toxicity; and (G) visualization tools.

Reference:

1. WIREs Comput. Mol. Sci, 6:147–172. doi: 10.1002/wcms.1240, 2016.

(B) Prediction of toxicity of a drug molecule/chemical using Laboratory Zebra fish

Zebra fish are the darlings of the fish world. These are small shoaling cyprinid fish. Zebra fish, play well with others, but bigger fish might eat them. Zebra fish are found in freshwater streams and slow-moving waters and the most species-rich vertebrate family.

Class: Actinopterygii **Order:** Carnivora

Family: Cyprinidae **Phylum:** Chordata

Genus: Danio **Sub Phylum:** Vertebrata

Scientific name: Danorerio rerio

For the last two decades Zebra fish has been widely used as experimental animals in biology, due to characteristics of it to enable embryological manipulations and large-scale genetic studies. Zebra fish are now a well established vertebrate animal model mostly used by developmental biologists for its easy observation and manipulation during embryogenesis. Zebra fish are also agreeable to genetic approaches and molecular manipulation *in vivo*. It can be a ‘‘model’’ for studying neural development and functions. The main advantage of this model is easy to understand the mechanisms of substance absorption, distribution, metabolism, and excretion. Hence, it is having wider application in toxicological screening of drugs and chemicals.

Zebra fish as a model for research on Cancer, tumour, cardiovascular disease, blood disorder. It is a good model for environmental toxicity study as well testing the toxicity of drugs and chemical at embryonic level.

Reference:

Dr.V.Parthasarathy and Dr.K.Vasudevan. Text Book “Laboratory Animals for Biomedical Research”. ISBN:

Prediction of toxicity of a drug molecule/chemical using cell biology techniques

The toxicity of drugs and chemicals can be tested using cell line, which is an alternative to animal study. It has several advantages, cost effective, less time consuming. The toxicity of the drugs and chemicals evaluated more appropriately by using specific cells. For an example the toxicity of chemicals on heart cell can be tested using heart cells. The toxicity of the drugs can be estimated using either MTT assay or SRB assay.

MTT Assay

Principle:

The MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay was the first homogeneous cell viability assay developed for a 96-well format that was suitable for high throughput screening (HTS). The MTT tetrazolium assay technology has been widely adopted and remains popular in academic labs as evidenced by thousands of published articles. The MTT substrate is prepared in a physiologically balanced solution, added to cells in culture, usually at a final concentration of 0.2 - 0.5mg/ml, and incubated for 1 to 4 hours. The quantity of formazan (presumably directly proportional to the number of viable cells) is measured by recording changes in absorbance at 570 nm using a plate reading spectrophotometer. A reference wavelength of 630 nm is sometimes used, but not necessary for most assay conditions.

Viable cells with active metabolism convert MTT into a purple colored formazan product with an absorbance maximum near 570 nm. When cells die, they lose the ability to convert MTT into formazan, thus color formation serves as a useful and convenient marker of only the viable cells. The exact cellular mechanism of MTT reduction into formazan is not well understood, but likely involves reaction with

NADH or similar reducing molecules that transfer electrons to MTT. Speculation in the early literature involving specific mitochondrial enzymes has led to the assumption mentioned in numerous publications that MTT is measuring mitochondrial activity.

The formazan product of the MTT tetrazolium accumulates as an insoluble precipitate inside cells as well as being deposited near the cell surface and in the culture medium. The formazan must be solubilized prior to recording absorbance readings. A variety of methods have been used to solubilize the formazan product, stabilize the color, avoid evaporation, and reduce interference by phenol red and other culture medium components. Various solubilization methods include using: acidified isopropanol, DMSO, dimethylformamide, SDS, and combinations of detergent and organic solvent. Acidification of the solubilizing solution has the benefit of changing the color of phenol red to yellow color that may have less interference with absorbance readings. The pH of the solubilization solution can be adjusted to provide maximum absorbance if sensitivity is an issue; however, other assay technologies offer much greater sensitivity than MTT.

The amount of signal generated is dependent on several parameters including: the concentration of MTT, the length of the incubation period, the number of viable cells and their and metabolic activity. All of these parameters should be considered when optimizing the assay conditions to generate a sufficient amount of product that can be detected above background.

MTT assay widely used for several toxicity testing of drugs for cancer, cardiovascular and neurological disorders etc.