Current approaches to toxicity profiling in early-stage drug development

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Abstract

The primary objective of this review is to explore the toxicological evaluation of new chemical entities. Toxicity testing of novel drugs plays a crucial role in the drug discovery and development process. This article highlights the various methods used for general toxicity assessment and provides an overview of toxicological profiling related to different diseases, with a focus on both in vitro and in vivo studies.

Keywords: Toxicological studies, Drugdiscovery, New drugs, *in-vitro, in-vivo* studies

1. Introduction

A New Chemical Entity (NCE) refers to a drug that contains an active moiety not previously approved by the United States Food and Drug Administration (USFDA) in any other application. Manufacturers generally highlight NCEs during the early phases of the drug development cycle, particularly as breakthrough candidates. At this stage, the compound undergoes extensive preliminary and clinical evaluation before being recognized as a drug.

The active moiety is defined as the part of the molecule responsible for its pharmacological or physiological action. This excludes appended portions of the molecule that render it an ester, salt (including salts with hydrogen or coordination bonds), or certain non-covalent derivatives such as complexes, chelates, or clathrates.

Given the potential risks and biological impact of new compounds, their development is strictly regulated. Unlike food products, which are primarily assessed for sensory attributes such as appearance, odor, and taste, new drug candidates require comprehensive preclinical toxicity evaluations before approval.

The development of a new drug, including an NCE, can be broadly categorized into four key stages:

- Drug discovery
- Preclinical development
- Clinical studies
- Marketing authorization, followed by post-marketing surveillance of drug-like molecules [1-3]

2. Methods of toxicity studies

a) Acute toxicity studies

Separate single-dose or high-intensity toxicity studies are generally no longer considered essential. Acute toxicity can usually be evaluated through short-term dose range-finding or dose-escalation studies, which may be conducted as non-GLP (Good Laboratory Practice) studies. The primary aim of acute toxicity studies is to identify potential target organs adversely affected by the test compound. Determination of the median lethal dose (LD_{50}) is no longer recommended. Extended single-dose toxicity studies may be performed to support early-phase clinical trials (especially first-in-human single-dose trials). These studies allow assessment of parameters such as hematology, clinical chemistry, and histopathology data.

b) Repeated-dose toxicity studies

Repeated-dose toxicity studies are conducted to characterize toxicological profiles, particularly to identify target organs and tissues affected by repeated administration of high doses. These studies also aim to establish no-effect levels, such as the No-Observed-Adverse-Effect Level (NOAEL), which serve as safety margins and help determine the highest dose to be used in subsequent toxicological evaluations. Such studies are required prior to First-in-Man (FIM) trials and support the initiation of clinical trials.

Studies must be performed in compliance with GLP and typically in two animal species: one rodent (rat or mouse) and one non-rodent (dog, minipig, or primate). Selection of the most appropriate animal model is essential to ensure translational relevance of preclinical findings to human trials. Ideally, the pharmacokinetic, pharmacodynamic, and metabolic profiles of the animal models should closely resemble those in humans.

c) Sub-chronic toxicity studies

In this study, 24 healthy albino rabbits (either sex, 1200–1800 g) were randomly divided into three groups. The control group received only DMSO, while the two treatment groups were administered 20 mg/kg and 60 mg/kg doses of the herbal

formulation orally for 60 consecutive days via intubation. During the 7-day acclimatization period, animals were monitored for physical health indicators such as hair loss, diarrhea, edema, ulceration, and reduced activity.

d) Sample collection

At the end of dosing (day 61), approximately 6 ml of blood was collected from each animal via cardiac puncture for biochemical and hematological analysis.

e) Physical examination

Gross signs of toxicity were observed weekly over the 60-day treatment period. Parameters included skin ulceration, weight variation, alopecia, loss of appetite, reduced activity, hematuria, vomiting, diarrhea, edema, lacrimation, salivation, muscle tone abnormalities, tremors, and aggressive behavior. At study termination, necropsy and organ dissection were carried out for further biochemical and histopathological analysis.

f) Biochemical evaluation

Prior to necropsy, blood samples (≈7 ml) were collected from fasting animals via cardiac puncture. Serum was separated by centrifugation (10 min, 4000 rpm) and analyzed within 3 hours using a Humalyzer 3000 (Human GmbH, Germany) at 37°C, with reagents provided by the manufacturer.

g) Hematological evaluation

Blood samples collected in EDTA tubes (10%, pH 7.2) were analyzed for hematological parameters including RBC, WBC, PLT, hematocrit, and hemoglobin using the Humacount Hematology Analyzer GmbH 17400 (with veterinary software module).

h) Microscopic examination

Tissue samples from heart, liver, and kidneys were excised, trimmed of fat, and processed using a Gilford 101 programmable tissue processor. Sections of 3–4 µm thickness were cut from paraffin blocks using a rotary microtome and mounted on glass slides. Slides were dried on a hotplate (45 °C, 1.5 h) and incubated briefly at 37 °C before histopathological evaluation.

i) Statistical analysis

Biochemical data were expressed as mean ± standard error of the mean (SEM). Statistical significance was determined using one-way ANOVA. A p-value <0.05 was considered significant, while $p < 0.005$ was considered highly significant.

3. Toxicological profiling of new chemical entities

a) Genotoxicity and carcinogenicity studies

These studies are commonly conducted in rodents (rats and mice) to assess potential genetic damage caused by new drug candidates. Genotoxicity refers to the ability of a substance to induce DNA or chromosomal damage through various mechanisms. Such alterations, including mutations in germ or somatic cells, may lead to permanent heritable changes and ultimately contribute to cancer development if not repaired by cellular mechanisms such as DNA repair or apoptosis.

A standard battery of *in vitro* and *in vivo* assays is recommended under ICH S2(R1) guidelines and OECD protocols. These include the bacterial reverse mutation test (Ames test, using *Salmonella typhimurium*), which reliably identifies mutagenic and carcinogenic compounds. Additional *in vitro* tests, such as the micronucleus assay or mouse lymphoma thymidine kinase (Tk) mutation assay, detect chromosomal and gene-level damage. Positive results from in vitro studies should be confirmed with *in vivo* assays to validate findings.

All genotoxicity and carcinogenicity studies must be conducted in accordance with GLP to ensure reliability and regulatory acceptance.

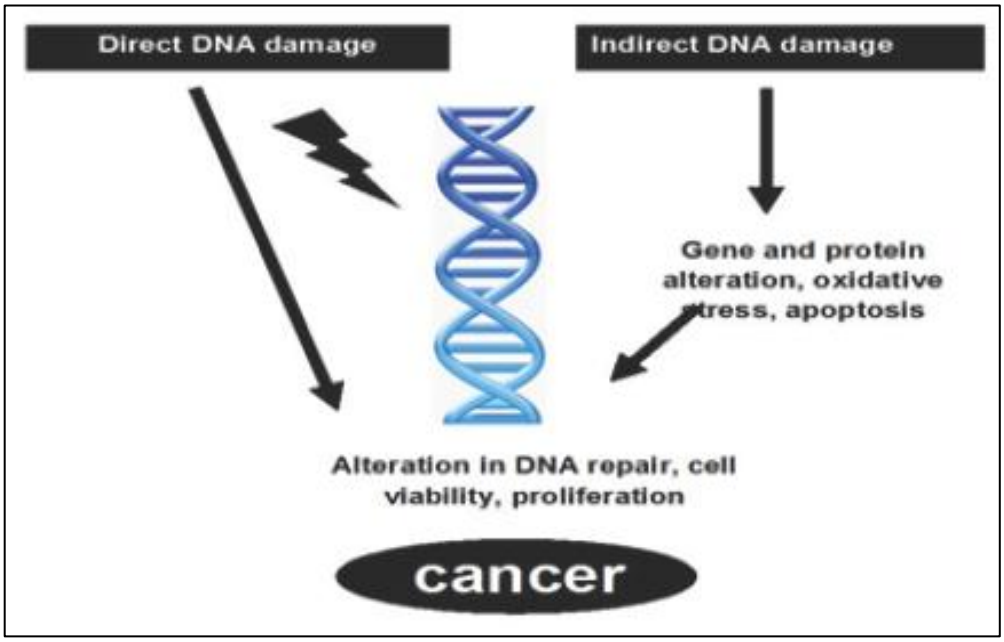

Fig 1: Relationship between carcinogenecity and Genotoxicity [16]

Dose descriptors and risk assessment of carcinogens

- It is widely recognized that certain chemicals, referred to as non-threshold carcinogens, may pose a cancer risk even at the lowest exposure levels. For such compounds, the traditional NOAEL (No-Observed-Adverse-Effect Level) and safety factor approaches are not suitable for establishing safe exposure standards.
- Two commonly applied dose descriptors in carcinogenicity assessment are T25 and BMD10, both of which are typically derived from two-year carcinogenicity bioassays in rodents (rats or mice).
- **T25**: The chronic daily dose that induces tumors in 25% of test animals at a specific site, adjusted for spontaneous incidence, over the natural lifetime of the species.
- **BMD10**: The benchmark dose estimated to produce tumors in 10% of the test population at a given tissue site, after adjustment for spontaneous occurrence, within the species' lifetime (17).

Acute inhalation toxicity testing

Acute inhalation toxicity testing is performed primarily for aerosolized or vaporized substances, with rodents being the preferred species. Animals are acclimatized to laboratory conditions (temperature 22 ± 2 °C, airflow 12–15 air changes per hour, and oxygen concentration ~19%). They are exposed to the test substance for at least 4 hours and monitored for a 14-day observation period.

- During exposure, food is withheld, while water may also be restricted under specific conditions.
- Clinical signs such as tremors, convulsions, salivation, diarrhea, lethargy, sleep, or coma are recorded.
- Mortality during both exposure and observation periods is documented.
- Necropsy is performed on deceased animals to identify histopathological and pathological changes. At study termination, surviving animals are euthanized for pathological evaluation (18).

Dose metrics for inhalation

- The standard dose descriptor for acute toxicity is LD50 (Lethal Dose 50%), representing the statistically derived dose expected to cause death in 50% of the test population. For inhalation studies, exposure is expressed as LC50 (Lethal Concentration 50%), defined as the concentration in air that causes mortality in 50% of test subjects.
- Units:
  - LD50: mg/kg body weight/day
  - LC50: mg/L air concentration
- LD50/LC50 values are primarily used for GHS acute toxicity classification, qualitative hazard evaluation, and dose selection in repeated-dose toxicity studies. They are not suitable for deriving NOAEL values (19).

Acute toxicity testing for topical preparations

Visual and dermal examinations play a crucial role in assessing acute toxicity of topical preparations. The Draize Eye Irritation Test and Draize Skin Irritation Test are the classical methods used to evaluate the potential hazards of chemicals and pharmaceuticals in rabbits and guinea pigs.

- **Eye irritation test:** 0.5 mL of the test substance is instilled into the conjunctival sac and exposure maintained for 4 hours. Observations include redness, swelling, discharge, ulceration, opacity, and blurred vision, monitored for up to 14 days.
- **Skin irritation test:** 0.5 g of the test substance is applied to the shaved skin surface of the animal. Reactions such as erythema and edema are evaluated during the 14-day observation period.
- Alternative *in vitro* methods are increasingly available to reduce or replace the Draize test for eye irritation.
- At the end of the observation period, animals are euthanized, and histopathological changes are assessed (20).

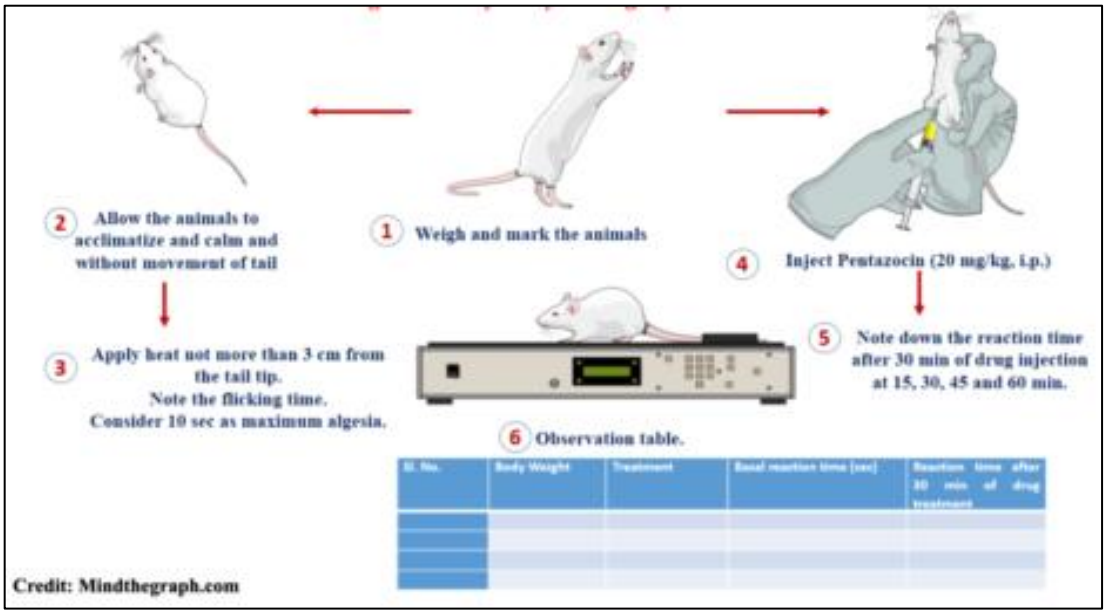

Fig 2: Acute toxicity testing for topical preparations [20]

Dose descriptor for skin/Eye irritation

The No Observed Adverse Effect Level (NOAEL) cannot be established from skin or eye irritation studies due to study design limitations. For these endpoints, qualitative hazard characterization is recommended, based on severity categories (e.g., corrosive, severe, mild, or non-irritant). Appropriate risk management measures (RMMs) should then be defined accordingly (21).

Reproductive toxicity studies

Reproductive toxicity encompasses both fertility and developmental toxicity. Fertility studies evaluate potential risks to male and female reproductive capacity, while developmental studies assess toxic effects on embryos, fetuses, and neonates. Adverse outcomes may include infertility, miscarriage, teratogenicity, or congenital malformations.

For such studies, mammalian species commonly used in pharmacological and toxicological research should be selected to ensure comparability with existing data. One rodent (typically rats) and one non-rodent species (commonly rabbits) are generally recommended. Repeated-dose toxicity data often provide relevant fertility information, especially regarding male reproductive health.

In addition to in vivo studies, in vitro models using tissues, organs, or cell systems can complement findings. Test substance administration should mimic the intended clinical route. Dose selection should be based on existing toxicological data; if unavailable, preliminary studies are necessary (22).

Dose descriptor for reproductive toxicity

- **NOAEL (No Observed Adverse Effect Level):** The highest exposure level without biologically significant adverse effects compared to controls.
- **LOAEL (Lowest Observed Adverse Effect Level):** The lowest exposure level at which adverse effects are observed.

  Both parameters are essential for quantitative risk assessment.

Subchronic oral toxicity test (90-day repeated-dose study)

Subchronic toxicity is evaluated in rodents and non-rodents following daily oral administration of the test substance for 90 days. Parameters include weekly body weight monitoring, monthly biochemical and cardiovascular assessments, and behavioral evaluations. At study termination, animals are sacrificed for gross pathological and histopathological examination.

To ensure reliability, variability among animals should be minimized, with acceptable body weight variation within ±20% (24).

Dose descriptor

- NOAEL and LOAEL values can typically be derived from repeated-dose studies.
- Units are expressed as mg/kg body weight/day or ppm; for inhalation studies, mg/L/6h/day is used (25).

Developmental toxicity / Embryotoxicity studies

Developmental and embryotoxicity can be assessed through in vivo and in vitro methods. Rodents are frequently used in vivo, with compounds administered during critical gestation periods (e.g., day 8–14 of pregnancy). At term, cesarean sections are performed to evaluate fetal outcomes such as resorptions, hemorrhage, congenital malformations, or mortality.

In vitro assays include the Embryonic Stem Cell Test (EST), micromass culture, and whole-embryo culture systems, which provide additional mechanistic insights (26).

Laboratory analysis of toxins

Toxins can be evaluated both qualitatively and quantitatively.

- **Qualitative methods:** Determine the nature of toxins using techniques like colorimetry and UV–visible spectrophotometry.
- **Quantitative methods:** Assess concentration and biochemical properties using advanced techniques such as infrared spectroscopy, gas chromatography, high-performance liquid chromatography (HPLC), and immunoassays (27).

Euthanasia of experimental animals

Euthanasia refers to the humane sacrifice of animals with minimal physical and psychological distress (28).

In biomedical research, euthanasia may be required:

- To prevent undue suffering when pain or distress exceeds acceptable levels, or when recovery is unlikely.
- To obtain biological material (enzymes, cells, tissues, or organs) for in vitro studies (29).

Zebrafish as a model in toxicology

The zebrafish (Danio rerio) is a widely used vertebrate model in drug discovery and preclinical research. Its regenerative capacity, sequenced genome, and availability of transgenic lines make it a valuable alternative to traditional mammalian models (30).

a) Zebrafish in Cardiotoxicology

Although structurally distinct, zebrafish hearts share key electrophysiological properties with humans. Their two-chambered heart exhibits similar action potentials, heart rate, and calcium signaling dynamics, making them suitable for cardiotoxicity screening.

Unlike rodents, zebrafish provide scalable, cost-effective, and genetically tractable systems for detecting drug-induced cardiotoxicity. Non-invasive imaging methods further enhance their utility in cardiovascular research (31).

b) Zebrafish in Hepatotoxicity

Zebrafish liver development begins by 3 days post-fertilization and becomes fully functional by 5 days. The liver exhibits mammalian-like functions, including metabolism of lipids, proteins, and xenobiotics, supported by diverse cytochrome P450 enzymes.

Histopathological liver damage, apoptosis, and biomarker expression in zebrafish parallel those seen in mammals. Transgenic fluorescent reporter lines (e.g., fabp10:RFP) enable real-time monitoring of liver injury.

The ZeGlobalTox assay now allows simultaneous assessment of cardiotoxic, neurotoxic, and hepatotoxic effects in zebrafish larvae, further supporting their role as an alternative model in drug safety studies (32).

Conclusion

This review highlights the importance of toxicological evaluation in new chemical entities (NCEs) as a critical step in drug development. Despite reliance on animal models for safety testing, translation to humans remains essential for establishing true safety and efficacy. Given the increasing reports of adverse drug effects, strengthening preclinical toxicological studies is vital for minimizing risks associated with novel therapeutics.

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