GENOTOXICITY TESTING: A REVIEW

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Abstract: Genotoxicity is a major limitation of various compounds used in clinical practices which range from gene mutations, chromosomal aberrations to other DNA effects. For the same reason, it must be assessed before bringing into the market. Moreover, various regulatory agencies have made it mandatory to submit the genotoxicity data while filing a New Drug Application (NDA). This in turn has given further importance to genotoxicity tests. This review deals with newer testing techniques used in genotoxicity in comparison with the established standard techniques.

Key words: Genotoxicity testing

INTRODUCTION

De Vries was the first to propose the concept of mutation, thereby laying down the foundations of genetic toxicology and has become clear that the stability of gene is not absolute and permanent changes can take place in the genetic material. [1] The new gene is called as a mutant and the process as mutation [2] Mutation [3] can be defined as any unexpected and undirected change in the composition of genetic information. The role of mutation [4] in drug resistance and carcinogenicity has made it an independent field of study altogether. Mutations [5] can arise spontaneously or under the influence of external factors, such as temperature, chemical substances, ultraviolet light and ionization radiation.

Towards the end of 1960’s, it was realised that people were increasingly being exposed to the mutagenic agents that could cause inheritable changes, and thus are transferred to the next generation. These mutations are responsible for genotoxicity which results in the increased incidence of cancer and genetic diseases. Genotoxicity can be defined as the amount of damage a genotoxin (toxin which affects DNA) can cause to a DNA molecule [6] which is studied under the genetic toxicology, a branch of toxicology. The development of genetic toxicology began in the midst of increasing awareness of the human exposure to toxic chemicals in the environment due to the modernization of life. These chemicals that produce the alterations in the nucleic acids and associated components at sub-toxic exposure levels, resulting in modified hereditary characteristics or DNA inactivation are termed as genotoxic agents [7]. There are three major types of genotoxic effects: gene mutations [8], chromosomal aberrations, and DNA effects. To detect these alterations in nucleic acids, genotoxicity tests are performed.

Genotoxicity tests can be defined as in vivo-in vitro tests designed to detect the compounds which induce genetic damage directly or indirectly by various mechanisms [9]. The validity of these tests depends upon the assumption that the DNA in different organisms is susceptible to chemical or physical damage to the same extent [10].

Various surveys revealed that the level of carcinogenicity of a compound depends upon the results obtained after performing genotoxicity tests on that compound. Most carcinogens [11] give positive results in routinely performed tests such as the salmonella assay and the chromosome aberration assays.
However, it must be noted that the certain carcinogens, like hormonally active compounds which can increase genotoxic activity without themselves being genotoxic, cannot be detected by short-term tests, which measure only the intrinsic genotoxic activity of a substance. As a result long-term assays like Rodent Lifespan Assay are required to be performed.

The tests that are performed are associated with some or the other drawbacks, the major one being time factor. Hence, various modifications have been done in the tests to increase the sensitivity and detection limit. This has given birth to newer methods of assessment of genotoxicity. As genotoxicity is a serious drawback of a compound, it must be assessed before bringing the drug in market. Various regulatory agencies have made it mandatory to submit genotoxicity data while filing a New Drug Application (NDA) [12]. As per the standard guidelines for genotoxicity, following tests are recommended [13].


Since no single validated test can provide information on all of the genetic endpoints and is incapable of detecting genotoxic effects like chromosome aberration, a battery of tests is usually recommended. Gene mutation and chromosomal aberration tests detect actual lesions in the DNA molecule, while DNA effects tests detect events that may lead to cell damage [15].

The three test battery, suggested by various regulatory agencies, is usually sufficient to detect the genotoxicity of a compound. Compounds giving positive results in the standard test battery may, depending on their therapeutic use and dosage regimen, need to be tested more extensively [16]. A genotoxic material has been defined as “an agent that produces a positive response, eg: mutagen, unscheduled DNA synthesis (UDS), and chromosome breakage, in any bioassay measuring any genetic endpoint” [17]. This definition is only a convenient method of classifying all chemicals into genotoxic or non-genotoxic groups. Test batteries may consist of screening tests, risk assessment tests or both. It is important at the outset of testing to carefully define the objectives desired in a test program [18].

Classification of the widely used assays [19]:

Bacterial mutagenesis assay: (Ames test) [19]:
This is the preliminary test performed to detect the carcinogenic potential of an entity using bacteria. This test detects the point mutation or frame shift mutation. In the Ames test, a strain of Salmonella typhimurium auxotrophic (deficient) for histidine (his), and which requires exogenous histidine, is used. Hence the bacteria are unable to survive in a medium devoid of Histidine.

This assay is used to detect mutation of the histidine gene (his) back to wild type (his+). As a result bacteria do not require exogenous histidine for growth. As this assay is used to detect the reverse mutation, it is also referred as “reversion assay” and the mutants are revertants selected on the agar deficient for histidine [20]. Majority of genotoxic agents can be detected by this test. It is based on the principle that most carcinogens induce cancer because they are mutagens. If these agents are shown to be mutagenic for bacteria, they may also alter DNA in eukaryotic cells. The best-validated strains are Salmonella typhimurium strains TA1535, TA1537 (or TA97 orTA97a), TA98 & TA 100. In order to detect some oxidizing mutagens and cross linking agents, Escherichia coli strains WP2 (pKM101), WP2uvrA (pKM101) or Salmonella typhimurium TA102 should also be used in the bacterial test. Escherichia coli assay is complementary to the Salmonella assay. This assay detects the frame shift of A – T mutation similar to salmonella assay but the gene is involved in the tryptophan biosynthesis and the tester strains are auxotrophic mutants (trp) and requires the tryptophan for the growth. For e.g., the genotoxicity of the antihypertensive agents
hydralazine and dihydralazine was tested in mammalian cells and bacteria. Both drugs elicited DNA repair in rat hepatocyte primary cultures. These observation support that hydralazine is carcinogenic in mice. The carcinogenicity of many chemicals results from interaction with DNA. Since these studies demonstrated that hydralazine and dihydralazine damage DNA in mammalian cells, these drugs can be viewed as potential human carcinogens [21] (Fig. 1).

Modification of Ames test using reporter genes:
Reporter genes have become an invaluable tool in studies of gene expression. They are widely used in biomedical and pharmaceutical research and also in molecular biology and biochemistry. A gene consists of two functional parts: One is a DNA-sequence that gives the information about the protein that is produced (coding region). The other part is a specific DNA-sequence linked to the coding region; it regulates the transcription of the gene (promoter). The promoter is either activating or suppressing the expression of the gene. The purpose of the reporter gene assay is to measure the regulatory potential of an unknown DNA-sequence. This can be done by linking a promoter sequence to an easily detectable reporter gene such as that encoding for the firefly luciferase. Common reporter genes are β-galactosidase, β-glucuronidase, and luciferase and to measure expressed reporter gene protein, the method of detection for expressed protein is based on the property of the protein e.g. luminescence is checked for the luciferase.

The most versatile and common reporter gene is the luciferase of the North American firefly Photinus pyralis. The protein requires no post translational modification for enzyme activity; it is not even toxic in high concentration (in vivo) and can be used in pro- and eukaryotic cells [22]. Luciferases are enzymes that emit light. The luciferase releases green light during the oxidation of its chemical substrate, luciferin. Other organisms, including plants that express the luciferase (LUC) gene will also glow faintly green when supplied with luciferin. The glow is widely used as an assay for LUC expression, which acts as a “reporter” for the activity of any regulatory elements that control its expression. Luciferase is particularly useful as a reporter in living cells and organisms. LUC gene fusions provide a “window” on to the mechanisms that regulate the activity of specific genes, in specific, living cells [23].

Salmonella typhimurium test uses the light emission of bacteria to detect the genotoxicity, cytotoxicity and mutagenic potency of the sample. It is used for the detection of genetic damage caused by the chemical in pharmaceutical research. It is a high throughput bacterial genotoxicity assay, which can detect DNA damage caused by genotoxic compounds. The assay is based on a reporter gene system where luciferase activity is used as a function of the genotoxicity. Luciferase expression is activated via a cascade of reactions known as the SOS DNA-repair system [4] which is induced by genotoxic compounds [24]. Two genetically engineered Salmonella typhimurium strains are used in this test system, TA104 recN2-4 (Genox strain) and TA104 pr1 (Cytox strain). The former strain carries a plasmid containing the bacterial luciferase operon (luxCDABE) of luminous bacteria Vibrio fischeri under transcriptional control of a recN promoter (recN2-4). The latter strain constitutively expresses the lux operon. Genotoxic compounds activate the recN promoter in the Genox strain, which results in transcriptional induction of the lux operon followed by the enhancement of light emission. The cytotoxicity of the compounds is simultaneously assayed with reference to the Cytox strain to identify the non-specific enhancement of light emission. Concomitant use of the Genox and the Cytox strains allows us to identify false positive results caused by non-specific light emission induced by other mechanisms, and not by the genotoxic effect [25, 26].

Benefits of the assay are that the entire DNA content of the cell functions as a target for the genotoxin to display its effect. Therefore, only a few micrograms of the sample are normally required for the assay. A cytotoxicity assay is also performed together with each sample to prevent false positive and negative
results. No cell growth is required for the genotoxicity detection, resulting in very short assay times. The simple test procedure involved may make the test easy to automate to improve the throughput.

FISH (Fluorescent in situ hybridization)[25] [26] is a cytogenetic technique which also can be used to detect and localize the presence or absence of specific DNA sequences on chromosomes. It uses fluorescent probes which bind only to those parts of the chromosome with which they show a high degree of sequence similarity. Probes are often derived from fragments of DNA that were isolated, purified, and amplified for use in the Human Genome Project. Such type of probes can be used for detection of the DNA fragments produced after DNA damage by genotoxic agent.

**In vitro assay for gene mutation in mammalian cells:** [19] The two standard In vitro assays are designed in which they utilise either cultured mouse lymphoma L5178Y cells or Chinese Hamster ovary (CHO) cells. Both the assays are designed to detect forward mutations at specific loci and are designated as follows. 1. Thymidine kinase +/- (TK +/-) mouse lymphoma mutation assay [27]. 2. Chinese Hamster Ovary (CHO)/hypoxanthine guanine phosphoribosyl transferase (HGPRT) mutation assay [28]. These are named according to their respective target genes TK and HGPRT. The protocols for the two assays differ somewhat but follow the same basic principles. The TK +/- mouse lymphoma mutation assay:

This assay utilizes a strain of mouse lymphoma cell that has been made heterozygous at the TK locus. These cells contain the TK enzyme which is responsible for incorporation of exogenous thymidine into the cells by the process of phosphorylation. Trifluorothymidine (TFT) is a selective agent which is used in this assay. TFT is taken up by the cells using the TK enzyme, as a result, the cells containing this TK enzyme become sensitive to the cytostatic and cytotoxic effects of TFT. Forward mutations [2] of single functional TK gene can result in the loss of TK functional activity. Thus the cells become TFT resistant. These mutant cells can be quantitated after an appropriate expression period by cloning in a soft agar medium supplemented with the selective TFT.

**CHO/HGPRT mutation assay:** It was designed to select for mutation [5] at the X chromosome linked and therefore hemizygous HGPRT gene is used. This HGPRT enzyme catalyses the phosphorylation of purines. The selective agent used in this assay 6-thioguanine (6TG) is also a substrate for this enzyme. Hence cells that retain the functional HGPRT enzyme are susceptible to cytotoxic effect of 6TG. Forward mutations that results in the loss of functional HGPRT gene render the cells resistant to 6TG.

Both assays detect point mutations involving base substitutions, deletions, frameshifts and rearrangements within the locus and thus can be used interchangeably for detection of these types of lesions. However the TK +/- mouse lymphoma mutation assay is able to detect clastogenic lesions involving multiple genes and multilocus deletions.

For CHO/HGPRT Mutation Assay, the following criteria are used as guidelines for interpretation of assay results. An assay is considered positive if the test compound produces a dose-dependent increase in mutant frequencies with at least two consecutive doses showing mutant frequencies elevated above 40 mutants per 10^6 clonable cells. If a single point above 40 mutants per 10^6 clonable cells is observed at higher dose, the assay will be considered equivocal (Fig. 2).

**Assay systems for chromosome damage [19]**

Cytogenetic assays permit the direct visualization of test article-induced chromosome damage and can be performed both in vitro and in vivo. After exposure of the cells to the test article, metaphases are evaluated microscopically for chromatid and chromosome-type deletions or rearrangements. Cytogenetic assays can be used for screening chemicals for clastogenic activity.

**In vitro methods:** The in vitro assay methods most frequently used for screening chemicals for clastogenic potential include Chinese Hamster cells (ovary, CHO or lung, CHL) or human peripheral blood lymphocytes (PBL) which are stimulated to divide using phytohemagglutinin [29]. After exposure of cells
to a test article, dividing cells are arrested in metaphase using a spindle inhibitor such as Colchicine. Metaphase cells are collected at a time selected to assure that cells are in the first metaphase after chemical exposure. The cells are observed under the microscope for any anomalies. The test article is considered to induce a positive response when the percentage of cells with aberrations is increased in a dose-responsive manner.

Statistical analysis of the percent aberrant cells may be performed using variety methods; one of the most common is Fisher’s Exact test. This test is used to compare pair-wise the percent aberrant cells of each treatment group with that of the solvent control. The Cochran-Amritage test has been recommended as a standard test for dose responsiveness [30].

In vivo methods: The two most frequently used endpoints for in vivo evaluation of clastogenic potential are the bone marrow metaphase assay and the bone marrow micronucleus test. Both assays may be conducted using either mice or rats. Intraperitoneal route of administration of the test and standard drug is preferred in the bone marrow metaphase assay.

i. Metaphase Analysis: In the bone marrow metaphase assay, dividing bone marrow cells are arrested in metaphase by an intraperitoneal injection of colcemid. Metaphase cells are collected at two or three time points after dose administration. As in the in vitro assay, sample times are selected to assure analysis of first-division metaphase cells, both nondelayed and delayed. Cells are evaluated microscopically for chromosomal aberrations as in the in vitro assay. Animals exposed to vehicle alone are used as negative controls and the spontaneous background rate is very low. The percent of damaged cells routinely seen in either rat or mouse bone marrow ranges from 0-2%. Positive controls are included in each assay, the most frequently encountered being cyclophosphamide or mitomycin C. (Table 1).

ii The test article is considered to induce positive response when the number of aberrant cells is significantly increased in a dose-responsive manner relative to the vehicle control. The test article is judged negative if no statistically significant increase in percent aberrant cells is observed relative to the control group at any sampling time. [31] For data analysis, the mitotic index and the total number and types of aberrations should be presented for each animal. As with the in vitro assay systems, gaps are presented in the data but they are not included in the total percent of cells with one or more aberrations or in the average number of aberrations [32] per cell. The percentage of damaged cells in the total population of cells scored is calculated for each treatment group. The severity of damage within the cells is reported as the average number of aberrations per cell for each treatment dose. Both male and female animals are dosed separately to analyze the test agent. Bone marrow micronucleus test is often used as a substitute for the more technically difficult and laborious bone marrow metaphase assay.

The micronucleus test is an in vivo assay for the detection of the both clastogens and agents that induce aneuploidy (abnormal chromosomal segregation i.e. nondisjunction). Although this test was initially developed using bone marrow erythrocytes of mice, it has also been conducted in rats, hamsters and monkeys. Yet, mice are the preferred species. It is used as a substitute for the technically more difficult and more laborious bone marrow metaphase assay [33].

Micronuclei arise from chromosomal fragments resulting from chromosomal breaks or detached chromosomes. Since micronuclei are formed by intact chromosomes or chromatid fragments which are not incorporated in to the nuclei of daughter cells during cell division, their presence is used to detect agents which are either clastogens or which alter integrity or function of the spindle apparatus. Due to the strong correlation between chromosomal breakage and micronucleus formation, the two assay systems are considered to be equivalent for screening purposes [34].

In the test, the target cells are the bone marrow erythroblasts. Chemically induced micronuclei in the erythroblasts are retained in the erythrocytes after the extrusion of the main nuclei from the cells maturation and can be scored in polychromatic erythrocytes (PCE). An increase of micronuclei in PCE indicates genotoxicity of the test agent. Both, male and female mice are dosed with the test agent, usually intraperitoneal injection but other routes of dosing are also acceptable.

The process of micronucleus test involves bone marrow smears which are prepared at two or three time points after dose administration, usually 24 and 48 hrs after exposure. A later point, usually 73 hrs, may also be included because it has shown to be optimal for only a limited number of clastogens [34].
Smears are evaluated microscopically for micronucleated polychromatic erythrocytes (MPCE). The ratio of polychromatic erythrocytes (PCE) to normochromatic erythrocytes (NCE) is also measured as an indicator of bone marrow toxicity.

In general, the mean incidence of MPCEs should not exceed 5/100 polychromatic erythrocytes (0.5%) in the negative (vehicle only) control. The test article is considered to induce a positive response if a dose-responsive increase in MPCE is observed and one or more dose levels are statistically elevated relative to the vehicle (negative) control at any sampling time. The test article is judged negative if no statistically significant increases in micronucleated polychromatic erythrocytes above the concurrent vehicle control values are observed at any sampling time.

For both the bone marrow metaphase assay and the micronucleus test [34-37], it is important to collect and analyze bone marrow samples at multiple time points [29,]. This is done to allow metabolic activation and because many clastogens cause substantial cell cycle delay, no single time is optimal. Multiple dosing and multiple harvests are required to capture the window of maximum micronuclei occurrence in bone marrow PCE. Micronuclei can also be detected in PCE in mouse peripheral blood. The advantage of the peripheral blood assay is that easy sampling and multiple sampling from the same animal for kinetic studies are possible. The peripheral blood micronucleus test [38], however, can be performed only in mice, but not in any other species yet studied, because the mouse is the only species in which micronucleated erythrocytes are not removed by the spleen, but persistent in the circulating blood (Fig. 3).

**DNA damage and repair assay [19]:** The unscheduled DNA synthesis (UDS) assay measures repairable DNA damages induced by the test agent. Monitoring unscheduled DNA synthesis in primary cultures of rat hepatocytes [11] presents several advantages over other cell types used to monitor possible interactions between a test and DNA. First, the target cells possess the ability to metabolize many promutagens/procarcinogens to their active form, thus eliminating the need for an exogenous source of metabolic activation. Second, rat hepatocytes in culture are nearly 100% non-dividing, so no metabolic blocks are needed to inhibit replicative DNA synthesis. Third, the target cells are epithelial in origin. Since most human cancers are carcinomas, an assay using epithelial cells to monitor genetic damage may be more relevant to the in vivo situation than a similar assay using fibroblasts. Metabolic activation inside the hepatocytes proximal to DNA is believed to enhance the sensitivity for the detection of DNA damages induced by short-lived genotoxic metabolites.

**a. In vitro UDS assay:** The use of primary hepatocytes obtained from young adult (6 to 12 week old) Sprague-Dawley or Fischer rats has been demonstrated to be sensitive to the DNA-damage activity of a variety of chemicals [38, 39]. Primary rat hepatocytes are isolated from the liver and are plated onto coverslips, the cells are washed with culture medium (for the UDS assay, the medium contains 10 μCi/ml tritiated thymidine, ³H-TdR), refed with serum-free culture medium and exposed to the test article for approximately 18-20hrs at 37 °C. The cells on coverslips are then processed for autoradiography. The cells are evaluated on the basis of incorporation of ³H-TdR into hepatocyte DNA, as evident by the presence of silver grains over the nucleus, presumably as a consequence of DNA repair. For each treatment group, a mean net nuclear grain count and standard deviation (SD), as well as the proportion of cells in repair are determined. Any mean net nuclear count that is increased by at least 5 counts over the solvent (negative) control is considered significant [38,40]. A test article is judged positive if it induces a dose-related increase with no less than one dose significantly elevated above the solvent control. A test article is considered negative if no significant increase in the net nuclear grain counts is observed. The percentage of cells in repair may also be used in making a final evaluation of the activity of the test article.

![Fig. 3: Assay system to detect chromosomal anomalies.](image)
b. In vivo - in vitro UDS assay: In light of the change in the profile of hepatocyte metabolism immediately on removal of the cells from the animal [39], the in vivo-in vitro UDS assay is designed to account for complex patterns of metabolic activation, detoxification, uptake distribution, and excretion of chemicals. The experimental design is based on procedures as described by Mirsalis et al. [39] and Butterworth et al.. The in vivo-in vitro UDS assay [41] is similar to the in vitro UDS assay, except that the test agent is administered to the animals. The assay is then conducted in cultured hepatocytes. [42] This assay is usually conducted in rats [43], but studies can also be done on mice.

Hepatocytes are isolated at two time points (2-4 hours and 12-16 hours) after the administration of test article as well as positive and negative (solvent) controls by intraperitoneal route. The collection of hepatocytes at two time points is an attempt to permit detection of maximum UDS response that occurs shortly after treatment (e.g., with dimethylnitrosamine or methyl methanosulfonate), as well as those occurring at 12-16 hours post-treatment (e.g., with 2-acetylaminofluorene or 2, 6-dinitrotoluene) [19].

Hepatocytes are isolated as per the procedure described for the in vitro UDS assay. Ninety to 180 minutes after plating, the cells are washed once in serum-containing culture medium and refed with serum-free medium containing \(^{3}\)H-TdR. Four hours later the radioactive medium is removed and the plates washed in serum-free medium plus 0.25mM thymidine. The cells are then incubated for 17-20 hours in culture medium plus 0.25mM thymidine. After incubation, the cells are processed for autoradiography and silver grain counting as per procedures described for the in vitro UDS assay. An increase of grain counts in the nuclear region indicates DNA repair.

Both the in vitro and in vivo-in vitro UDS assays are capable of detecting liver carcinogens, but not necessarily carcinogens that affect other tissues. The in vivo-in vitro UDS assay is considered to be more reliable than the in vitro UDS assay, as the in vitro UDS assay is known to produce false-positive results, relative to rodent carcinogenicity (Fig. 4).

Transgenic assay for in vivo mutagenesis: An in vivo mutation system has been developed using transgenic mice [44]. Transgenic C57BL/6 and B6C3F1 mice have been constructed such that each cell of every tissue contains multiple copies of bacteriophage lambda vector at an identical chromosomal integration site. The lambda shuttle vectors contain a lacI gene as a target for mutagenesis testing. The shuttle vector carrying the target gene is recovered from genomic DNA using an in vitro packaging extract. The packaging extract contains enzymes which recognize and excise the shuttle vector from within the genomic DNA and which mediate the packaging into infectious phage particles. Each packaged phage represents a single rescued target gene. Packaged phages are plated with E. Coli cells and form plaques on the bacterial lawn.

The lacI gene was selected as the target gene because its mutations are easily detected, it is highly sensitive to mis-sense mutations, and it has been widely studied as a target for in vitro mutagenesis. The lacI gene codes for the production of the Lac repressor protein. This repressor protein binds to the lac operator and blocks the transcription of the lacZ reporter gene. Mutation in the target gene will render the resulting Lac repressor protein inactive, the lacZ gene will be expressed, and its protein product, β-galactosidase, will be produced. For detection of mutants using color screening system, a chromogenic substrate, X-Gal, is included in the plating agar. The β-galactosidase produced in mutant plaques appears blue in color. Plaques formed from the phage carrying non-mutant target genes produce an active Lac repressor protein which suppresses the production of β-galactosidase and are colorless. The transgenic mouse mutation system is highly adaptable to screening for mutagenic potential [44]. Mutation analysis can be preformed on any organ or tissue. [45] Mutational spectrum analysis also permits a detailed mechanistic analysis of mutagenesis at the molecular level. The mutant frequency is determined for each animal and tissue analyzed as the ratio of mutant to non-mutant plaques. A test article is considered to...
Sensitivity of *lacI* transgenic mouse mutagenicity assay [46]: The detection limit of the *lacI* transgenic mouse mutagenicity assay lies, in practice, at approximately a 50-100% increase in mutant frequency in treated animals over controls. The sensitivity of this assay in detecting genotoxins can be markedly improved by subchronic rather than acute application of the test compound. The *lacI* transgenic mouse mutagenicity assay was compared quantitatively to rodent carcinogenicity tests and to presently used *in vivo* mutagenicity assays. With the genotoxic carcinogens tested thus far, a rough correlation between mutagenic potency and carcinogenic potency was observed: on average, to obtain a doubling in *lacI* mutant frequency the mice had to be treated with a total dose equal to 50 times the TD50 daily dose level. This total dose could be administered either at a high dose rate within a few days or, preferably, at a low dose rate over several weeks. This analysis also indicated that a *lacI* experiment using a 250-day exposure period would give a detection limit approximately equal to that of a long-term carcinogenicity study. In comparison to the micronucleus test or the chromosome aberration assay, acute studies with the presently available *lacI* system offered no increase in sensitivity. However, subchronic *lacI* studies (3-4-month exposure) resulted in an increase in sensitivity over the established tests by 1-2 orders of magnitude (shown with 2-acetylaminofluorene, N-nitrosomethylamine, N-nitrosomethylurea and urethane). It is concluded that a positive result in the *lacI* test can be highly predictive of carcinogenicity but that a negative result does not provide a large margin of safety.

**Current status of genotoxicity:** The growth of new chemical entities is fast; hence the current scenario is demanding the improvement in methodologies and short term tests [47] [71]. The approach to evaluate the carcinogenic potential of pharmaceuticals has undergone important changes with the increasing knowledge of the mechanisms of carcinogenesis, available data of carcinogenicity studies, and technological progress [48].

The test batteries used conventionally are found to be inefficient in detecting accurately the genotoxic potential of the newer agents due to limitations such as: 1. The lengthy time span required to obtain results, 2. Incidences of false negative results and 3. Difference in the extent of toxicity *in vitro* and *in vivo*. As a result, it has become imperative to develop more sensitive methods of assay. To overcome these disadvantages the newer methods are developed on the following lines: a. Modification of the original test to increase the detection limit and decrease the time required. b. Use of computer assisted studies like QSAR technologies [49] and structure activity relationships [50, 51] that give structural alerts regarding the possible genotoxic effect. The latest advancement in the techniques predict the genotoxicity / carcinogenicity in a very short span of time [52] including assays such as Luciferase enzyme assay which gives test results within few hours.

**Alkaline comet assay [53,72,73]:** The *in-vivo* Comet assay has gained significant support as an alternative supplementary *in vivo* study as this test is not organ-specific unlike other genotoxicity tests. The *in vivo* Alkaline Comet assay can be used to detect various types of DNA damage induced by different classes of genotoxic compounds. The basic principle of the alkaline Comet assay is the migration of DNA in an agarose gel under an electrophoretic current. Fragments of damaged DNA move faster within the gel than intact DNA resulting in the microscopic appearance of the nucleus as a comet-like shape: The intact DNA in the nucleus forming the “head” and the small DNA fragments appearing as a “tail”.

The alkaline conditions for lysis and electrophoresis enable the visualization of single strand, as well as double strand DNA breaks. The main advantages of the Comet assay are its relatively easy application to almost any tissue of interest and the evaluation of different organs from the same animal. [74] E.g.
Comparison of the *in vitro* genotoxicity of anticancer drugs Idarubicin and mitoxantrone [54]. E.g. Assessment of the cytotoxic and genotoxic potential of 5-aminolevulinic acid on lymphocytes [55].

**Genotoxicity test system based on p53R2 gene expression in human cells** [75]: DNA damage induced by gamma-ray, ultraviolet irradiation and also by genotoxic chemicals such as adriamycin, activates the gene p53R2, which is responsible for encoding a subunit of ribonucleotide reductase. For the purpose of constructing an easy-operating genotoxicity test system using human cell lines, a p53R2-dependent luciferase reporter gene assay is developed, and dose-dependent luminescence caused by adriamycin in two human cell lines that express wild-type p53, MCF-7 and HepG2 is checked. On treating the cells having the modified p53R2 with the test sample of drug, which are previously tested to be Ames Positive or negative, the luciferase activity, i.e. the luminescence is measured. Results showed that a few of the Ames-negative drugs were showing positive results for genotoxic potential. Further, these drugs were confirmed to be genotoxic in other in vitro test systems using mammalian cells. It can be thus concluded that this assay system can be applied to rapid screening of chemicals for potential human genotoxicity as it shows better sensitivity as compared with the older assays such as the Ames test [56].

**The Combined bacterial SOS [11] – Lux and lac – fluoro test system** [76,77]: This test is the combination of two bioassays that simultaneously measure the genotoxicity (SOS Lux test) and the cytotoxicity (Lac fluoro test) of a substance and mixture of substances. The SOS – Lux assay is based on the genetically modified *Salmonella typhimurium* TA1535 bacteria which have been transformed with the plasmid pPLS -1 carrying the promoterless Lux gene of *Photobacterium leiognathi* as a reporter element under the control of DNA damage dependant SOS promoter from ColD as a sensing element. This system reacts with the production of the bioluminescence in a dose dependant manner to agents, which induce the DNA damages inside these bacteria. The SOS Lux Test as a bioassay for genotoxicity can be used partly or fully automatically for routine measurements and can be employed for high throughput screening. This bioassay uses the receptor – reporter principle with the SOS promoter system as preceptor sensitive to the DNA damage and controls the bioluminescence system as a reporter, emitting the bioluminescence light as a optical signal which can be recorded by an appropriate detector [57].

**Microscale Fluorometric Assay of DNA Unwinding (µ-FADU) [78]**: The Fluorometric Analysis of DNA Unwinding (FADU) assay was originally designed for rapid detection of X-ray-induced DNA damage in mammalian cells. This cellular bioassay principle is based on time-dependent alkaline denaturation of DNA under moderate denaturing conditions (pH 12.2-12.4) starting from ends as well as from all DNA break points (single-strand breaks, SSB; double-strand breaks, DSB; alkali-labile sites, ALS). DNA which remains double stranded after 30 min of alkaline treatment is detected after neutralizations and immediate fragmentation followed by binding to the Hoechst 33258 dye (bisbenzimide) and measuring its fluorescence.

The same method can be used to detect DNA damage due to certain chemicals. Here, DNA damage is detected after exposure of Chinese Hamster Ovary (CHO) cells with model substances at ice temperature revealing the methods suitability [58]. The method involves cell cultivation and chemical treatment in the same microplate followed by analysis of test samples in a microplate fluorescence reader. The mammalian cells are exposed to chemicals for 60 min at ice temperature thus allowing identification of direct acting substances capable of DNA strand break induction. An internal standard used in every plate is hydrogen peroxide. The ji-FADU approach is suitable to identify strand-break inducing chemicals within 2 hours. The microscale fluorometric analysis of DNA unwinding (ji-FADU) is a fast, sensitive and reliable method for the detection of strand breaks in DNA as an index of DNA damaging potential of chemical agents. DNA damage is measured as strand scission factor for unwound DNA after alkaline treatment.

**Development of the newer molecules and QSAR**: Decades of mutagenesis and clastogenesis studies have yielded enough structure-activity-relationship (SAR) [59] information to make feasible the construction of computational models for prediction of endpoints based on molecular structure and reactivity. Often a compound’s biological activity is determined by complex relationships between its structural components. Such a relationship often can only be adequately described and exploited by multivariate structure-activity relationship studies that can deal with many variables simultaneously [60].
The ability to predict genotoxicity of novel chemical entities computationally, thus precluding the necessity for conducting biological assays, might ultimately be achievable but we’re not there yet [51]. SAR approaches to the study of genotoxic phenomena are finding increased applications. However, the data being modeled are frequently not considered optimal due to the small size of the dataset and an uneven distribution of genotoxicants and non-genotoxicants in the dataset. Overall, the present analyses did not result in the development of SAR models of greatly increased predictivity. Conceivably, for the particular datasets and SAR paradigm the limit of predictivity has been reached. The possibility of investigating the use of a “battery” of SAR paradigms should be considered [50].

Apart from testing on drug substances, it has become necessary to also test other aspects that may contribute to the overall genotoxic potential of a drug formulation. This includes testing of drug metabolites, impurities and hazards of drug interactions which may end up in increasing the genotoxic threat of the formulation. Testing of drugs to be administered in pregnant women should also be given due consideration owing to potential hazards to the foetus.

Testing of the metabolites: Current research has been diverted to and demands the testing of metabolites of the chemical entity, especially the metabolites observed in the human. The extent of human exposure, structural knowledge of the metabolite, and metabolic activation are considered in defining the timing of pharmaceutical ADME assessments. Testing of the metabolites has two proactive approaches, which emphasize early metabolism predictions to drive appropriate hazard assessment; and a retroactive approach to manage safety risks of a unique or major metabolite once identified and quantitated from human clinical ADME studies. This has been realized by regulatory authorities and hence it has been made mandatory to test the metabolites of drugs which are used in long term therapy [61].

Genotoxicity and pregnancy: A due care is necessary during the pregnancy as the foetus is growing, all the drugs taken must be checked for their adverse effects as well as for genotoxicity. Foetal cells are rapidly growing and are responsible for tissue formation so if any kind of DNA damage occurs that would continue in all the cells. Such drugs would be highly toxic to foetus so they should be avoided during pregnancy. In general, drugs, unless absolutely necessary, should not be used during pregnancy because many can harm the fetus e.g. phenytoin a common anticonvulsant is found to be genotoxic [62]. Other drugs, like nimesulidin [63] and fansidar (antihypertensive)[64] are genotoxic and risky in pregnancy.

Impurities and genotoxicity: Impurities which are of genotoxic nature are of prime concern [65]. The impurities can arise from a variety of sources during the manufacturing process which include- residues from starting materials, byproducts, intermediates, reagents, legends and catalysts, as well as the degradation products arising during storage [66]. The various statutory bodies across the globe are working on the process of preparing guidelines to define acceptable limits of these impurities in new drug substances and novel excipients which have been found to be genotoxic. According to existing ICH Q3A(R) guideline on impurities in new drug substances, impurity acceptance criteria should be set no higher than the level that can be justified by safety data [67,68].

As per the regulatory point of view genotoxic compounds are usually considered to operate by a non-threshold mode of action and thus any level of exposure carries – at least theoretically – a risk. This further view implies that genotoxicity measurements should be guided by the so-called ‘As Low As Reasonably Achievable [69] ‘ALARA’ principle, that is where avoidance is possible genotoxic impurities must be kept to a level. In the last decade, the characterisation of many proteins involved in sensing and responding to DNA damage has enhanced our understanding of genotoxic stress responses. Mutations in the genes that encode DNA damage response proteins can result in a number of genomic instability syndromes, disorders that often result in a heightened predisposition to various types of cancer. Another hallmark of these disorders is immunodeficiency, a phenotype caused by an inability to properly repair DNA strand breaks that occur during development of the immune system. The phenotypes exhibited by genomic instability syndromes [70] highlight the significance of proteins that sense, relay, or transduce signals associated with the genotoxic stress response.

Though the newer methods with certain modifications...
are coming up, but the older ones cannot be ignored. The alternative approach in line with the development of models of genetically-modified mice that are conceived on the basis of known mechanisms of carcinogenesis are gaining the top priorities such models have also been evaluated through studies sponsored by the International Life Sciences Institute.

CONCLUSION

Genotoxicity testing of the drugs and their formulation is undergoing the revolution. Newer methods are getting developed and are assisting the older ones with their increased sensitivity and other advantages. The current scenario is also demanding the testing of excipient and formulation, metabolites, impurities and effects during pregnancy. Newer methods that are developed may be used for preliminary screening for genotoxicity yet they need development on the basis of quantization and the other aspects.

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