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Review Article

Genotoxicity of Drugs: Mechanisms, Testing Guidelines and Methods for Evaluation

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ABSTRACT

It is estimated that 80% of world population rely on traditional herbal medicine for primary health care. With the rising utilization of herbal products, safety and efficacy of herbal medicine have become a public health concern. Adverse health effects associated with herbal products could be attributed to both inherent toxic effects of herbal medicine and toxicities induced by adulterants.Although often perceived as innocuous by the general public, many herbs phytochemicals that are either directly reactive towards DNA or likely to disturb cellular homeostasis, cell cycle, andr genome maintenance mechanisms; this may translate into genotoxicity, carcinogenicity, or co-carcinogenicity. Genotoxicity refers to the deleterious effect of a chemical compound or a physical event on the genetic material; such genotoxic events are considered hallmarks of cancer risk. The numerous genome maintenance mechanisms of the cell and may not lead to cancer. The long-term safety evaluation is probably better investigated through carcinogenicity, which denotes the capacity of a chemical substance or a mixture of chemical substances to induce cancer or increase its incidence.

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Introduction

In genetics, genotoxicity describes the property of chemical agents that damages the genetic information within a cell causing mutations, which may lead to cancer. While genotoxicity is often confused with mutagenicity, it is important to note that all mutagens are genotoxic, however, not all genotoxic substances are mutagenic. The alteration can have direct or indirect effects on the DNA: the induction of mutations, mistimed event activation, and direct DNA damage leading to mutations. The permanent, hereditary changes can affect either somatic cells of the organism or germ cells to be passed on to future generations. Cells prevent expression of the genotoxic mutation by either DNA repair or apoptosis; however, the damage may not always be fixed leading to mutagenesis. Genotoxicity is a word in genetics defined as a destructive effect on a cell's genetic material (DNA, RNA) affecting its integrity. Genotoxins are mutagens; they can cause mutations. Genotoxins include both

radiation and chemical genotoxins. A substance that has the property of genotoxicity is known as a genotoxin. (IV)

Toxic substances which directly shows their impact on cell viability is for the most part mainly referred to as cytotoxins. All the chemicals which produce genetic knock out leading to mutation are known as genotoxic. Further, some classes of substances which are capable of damaging and interacting the genome within chemical genotoxins. Genotoxins include both radiation and are genotoxic (*NCBI*, 2020).

Genotoxins can be of the following category depending on its effects;

1. Carcinogens or cancer-causing agents.
2. Mutagens or mutation is causing agents.
3. Teratogens or congenital disability is causing agents.

Hence, genotoxicity can be described as the capacity of a substance to cause damage to the genetic information inside the cell. This DNA damage could result in mutations, thus promoting carcinogenesis or establishing the framework for congenital disorders. The damage which is caused by agents of genotoxins may also involve in direct interaction with the DNA, and resulting either in the base substitutions, frame-shift mutations, and also even double-stranded breaks. In some other cases, the substances which are genotoxic may also interact with various types of proteins are either engaged with replication or maintaining chromo-somal stability. Toxicological studies have encountered a compelling evolution during the previous decade, with enough outstanding emphasis being placed on chronic toxicity, teratogenicity, carcinogenicity, mutagenicity, etc. (V)

Mechanism

One of the endpoints of genotoxicity is gene mutations. Mutagenic chemicals cause predominantly gene mutations, which are generally not lethal but can form a major threat to the integrity of chromosomes and viability of cells. Depending on the specific classes of DNA lesions, one or more DNA repair pathways become active. Four of the 4 major DNA repair pathways are involved in the repair of DNA lesions leading to gene mutation that are as follows, (EMEA, 2006)

1. Direct pair
2. Base excision pair
3. Nucleotide excision pair
4. Mismatch repair

Direct Repair

Direct repair acts by removing or reversing the DNA lesions. These lesions can occur due to alkylating agents. Direct repair is carried out by specific enzymes called alkyl guanine-DNA methyltransferases (AGMT), which remove the alkyl group from the guanine residue of DNA and transfers it to one of its cysteine residues. Alkylating agents are reactive compounds that can transfer methyl or ethyl groups to a DNA base, thereby chemically modifying the base. A particularly important type of damage is methylation of the O6 position of guanine, because the product, O6-methylguanine, forms complementary base pairs with thymine instead of cytosine. This lesion can be repaired by an enzyme (called O6-methylguanine methyltransferase) that transfers the methyl group from O6-methylguanine to a cysteine residue in its active site (OECD, 1981).

Only a few types of DNA damage are repaired in this way, particularly pyrimidine dimers resulting from exposure to ultraviolet (UV) light and alkylated guanine residues that have been modified by the addition of methyl or ethyl groups at the O6 position of the purine ring. UV light is one of the major sources of damage to DNA and is also the most thoroughly studied form of DNA damage in terms of repair mechanisms. The major type of damage induced by UV light is the formation of pyrimidine dimers, in which adjacent pyrimidines on the same strand of DNA are joined by the formation of a cyclobutane ring resulting from saturation of the double bonds between carbons 5 and 6 (Mohamad, S. *et al.*, 2017).

Base excision repair

In excision repair, the damaged DNA is recognized and removed, either as free bases or as nucleotides. The resulting gap is then filled in by synthesis of a new DNA strand, using the

undamaged complementary strand as a template. The repair of uracil-containing DNA is a good example of base-excision repair, in which single damaged bases are recognized and removed from the DNA molecule. Uracil can arise in DNA by two mechanisms: (1) Uracil (as dUTP [deoxyuridine triphosphate]) is occasionally incorporated in place of thymine during DNA synthesis, and (2) uracil can be formed in DNA by the deamination of cytosine. The excision of uracil in DNA is catalyzed by DNA glycosylase, an enzyme that cleaves the bond linking the base (uracil) to the deoxyribose of the DNA backbone. This reaction yields free uracil and an apyrimidinic site—a sugar with no base attached. (EMEA, 2006)

Nucleotide-excision repair

The result of DNA glycosylase action is the formation of an apyridiminic or apurinic site (generally called an AP site) in DNA. Similar AP sites are formed as the result of the spontaneous loss of purine bases, which occurs at a significant rate under normal cellular conditions. For example, each cell in the human body is estimated to lose several thousand purine bases daily. These sites are repaired by AP endonuclease, which cleaves adjacent to the AP site. The remaining deoxyribose moiety is then removed, and the resulting single-base gap is filled by DNA polymerase and ligase. Whereas DNA glycosylases recognize only specific forms of damaged bases, other excision repair systems recognize a wide variety of damaged bases that distort the DNA molecule, including UV-induced pyrimidine dimers and bulky groups added to DNA bases as a result of the reaction of many carcinogens with DNA. (Savale, S.K., 2018)

Mismatch repair

The importance of this repair system is dramatically illustrated by the fact that mutations in the human homologs of MutS and MutL are responsible for a common type of inherited colon cancer (hereditary nonpolyposis colorectal cancer, or HNPCC). HNPCC is one of the most common inherited diseases; it affects as many as one in 200 people and is responsible for about 15% of all colorectal cancers in this country. The relationship between HNPCC and defects in mismatch repair was discovered in 1993, when two groups of researchers cloned the human homolog of MutS and found that mutations in this gene were responsible for about half of all HNPCC cases. Subsequent studies have shown that most of the remaining cases of HNPCC are caused by mutations in one of three human genes that are homologs of MutL (EMEA, 2006; Mohamad, S. *et al.*, 2017).

Guidelines

OECD Guidelines

For the Testing of Chemicals (OECD TG) are a set of internationally accepted specifications for the testing of chemicals decided on by the Organisation for Economic Co-operation and Development (OECD). They were first published in 1981. They are split into five sections:

Section 1: Physical Chemical Properties
Section 2: Effects on Biotic Systems.
Section 3: Environmental Fate and Behaviour.
Section 4: Health Effects
Section 5: Other Test Guidelines.

ICH Guidelines

This guidance replaces and combines the ICH S2A and S2B Guidelines. The revised guidance describes internationally agreed upon standards for follow-up testing and interpretation of positive results in vitro and in vivo in the standard genetic toxicology battery, including assessment of no relevant findings. This guidance is intended to apply only to products being developed as human pharmaceuticals (Kamath, G.H. *et al.*, 2013; Gallowa, S.M., 2017).

SCHEDULE Y

In the Drugs and Cosmetics Rules, 1945 (hereinafter referred to as said rules), (1) in Part X-A, after rule 122-DA, the following shall be inserted, namel,

122-DAA.: Definition of Clinical trial.- For the purpose of this Part, "Clinical trial" means a systematic study of new drug(s) in human subject(s) to generate data for discovering and / or verifying the clinical, pharmacological (including pharmacodynamic and pharmacokinetic) and /or adverse effects with the

objective of determining safety and / or efficacy of the new drug.

In the said rules for Schedule Y, the following Schedule shall be substituted, namely; SCHEDULE- 'Y' (122A, 122B, 122D, 122DA, 122DAA and 122E) (Shah, S.U., 2016; Gallowa, S.M., 2017)

Standard Test Battery for Genotoxicity

There are two fundamental areas in which harmonization of genotoxicity testing is considered necessary is

1. Identification of a standard set of tests to be conducted for registration.
2. The extent of confirmatory experimentation in *in-vitro* genotoxicity tests in standard battery.In general, the three standard genotoxicity test battery is adequate for evaluation of genotoxicity of NCE (New Chemical Entities).

S2A: Genotoxicity Guidance on specific aspects of regulatory genotoxicity tests for pharmaceuticals.

S2B: Genotoxicity: A standard battery for genotoxicity testing of pharmaceuticals.

S2A Guideline: The S2A guidelines cover the strategic issues and protocol design for *in-vitro* and *in-vivo* genotoxicity test.

The standard test battery for genotoxicity recommends the following for genotoxicity Evaluation (Shah, S.U., 2016; EMEA, 2006).

Table 1: Standard Test for Genotoxicity Evaluation

TG 471	Bacterial Reverse mutation test (Ames test)
TG 472	Genetic toxicology; Escherichia coli, reverse assay
TG 473	In-Vitro Mammalian Chromosome Aberration
TG 474	Mammalian Erythrocyte Micronucleus
TG 475	Mammalian Bone Marrow Chromosome Aberration
TG 476	In-Vitro Mammalian Cell Gene Mutation
TG 477	Genetic Toxicology: Sex-linked Recessive Lethal Test in Drosophila melanogaster.
TG 478	Genetic Toxicology: Rodent Dominant Lethal Test
TG 479	Genetic Toxicology: In-Vitro Sister Chromatid Exchange Assay in Mammalian cells.
TG 480	Genetic Toxicology: Saccharomyces cerevisiae, Gene
TG 481	Genetic Toxicology: Saccharomyces cerevisiae, Mitotic recombination Assay.
TG 482	482 Genetic Toxicology: DNA Damage and Repair, Unscheduled DNA synthesis in mammalian cells In-Vitro.
TG 483	Mammalian Spermatogonial Chromosome Aberration Test
TG 484	Genetic Toxicology: Mouse Spot Test
TG 485	Genetic Toxicology: Mouse Heritable Translocation Assay
TG 486	Unscheduled DNA Synthesis (UDS) Test with Mouse Liver cells In-Vitro.
TG 487	In-Vitro Mammalian Cell Micronucleus Test.

Testing for gene mutation in bacteria: In-vitro: cytogenetic evaluation of chromosomal damage with mammalian cells or mouse lymphoma assay.

In-vivo: test for chromosomal damage using rodent hematopoietic cells.

In-Vitro testing methods

Ames test (Bacterial reverse mutation test)

Bacteria: 1. *Salmonella typhimurium*: TA1535; TA1537 or TA97a or TA97; TA98 and TA100.

Procedure- There are two methods; 1. Plate incorporation method 2. Pre incubation method

Suspensions of bacterial cells are exposed to the test substance in the presence and in the absence of an exogenous metabolic activation system. Treatment mixture is incubated and then mixed with an overlay agar plating onto minimal medium. After two or three days of incubation, revertant colonies are counted and compared (Fig. 1).

Principle- After identifying the mutation it reverts it back and restores the functional capability of the mutant cell to synthesize Histidine. In this test the reverent bacteria cells are identified by the ability of the parent test strain to grow in the absence of amino acids. The bacterial reverse mutation test being rapid, inexpensive and easy to perform is commonly used as an initial screening test for genotoxicity or mutagenicity (OECD, 1981).

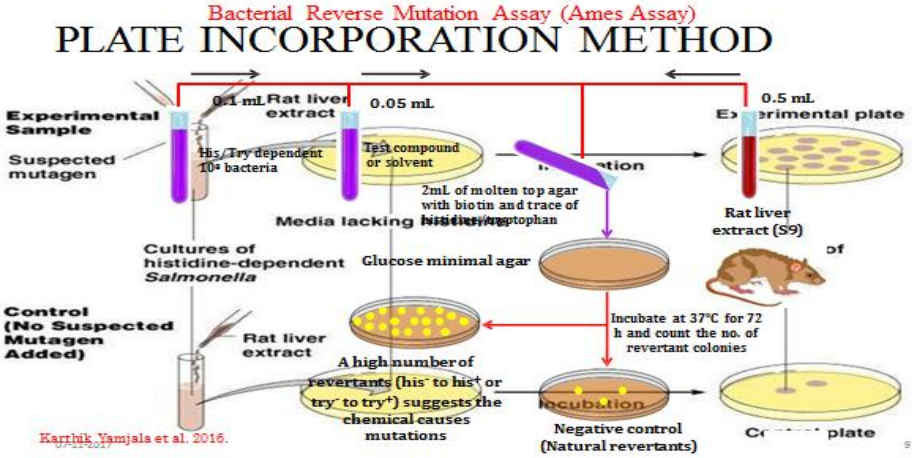

Fig. 1: Ames Test

TG487: INVITRO MAMALIAN CELL MICRONUCLEUS TEST-2010

Principle- detection of the frequency of micronuclei.

Procedure- Cell cultures of human or other mammalian origin are exposed to the test chemical.. Harvested and stained interphase cells are analysed.Treated with a cytokinesis blocker; this is easily achieved by scoring only binucleate cells. Assay detects the activity of clastogenic and an eugenic chemicals (Fig. 2) (NCBI, 2020).

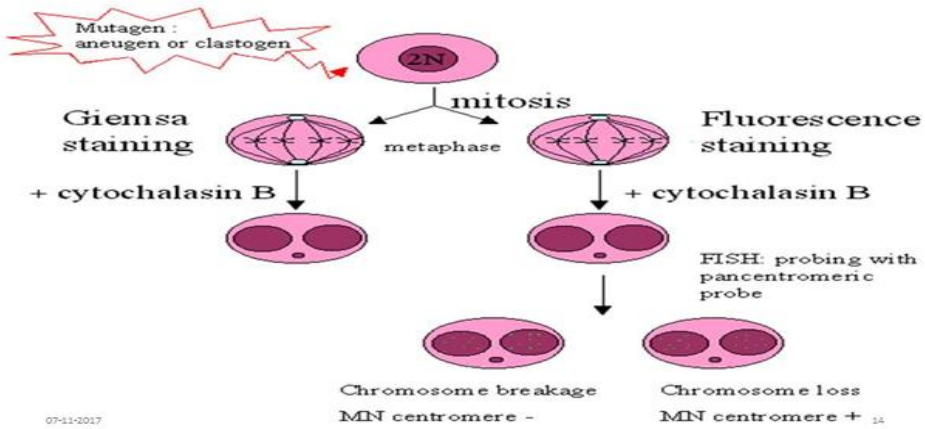

Fig. 2: In-Vitro Mammalian Cell Micronucleus Test 2010

TG 474: Mammalian Erythrocyte Micronucleus Test

Principle- Detection of damage induced by the test substance to the chromosomes or the mitotic apparatus of erythroblasts, by analysis of erythrocytes as sampled in bone marrow and/or peripheral blood cells of animals.

Procedure- Bone marrow = the animals are sacrificed, bone marrow extracted, and preparations made and stained. Peripheral blood = the blood is collected at appropriate times after treatment and smear preparations are made and stained.

Take number and sex of animals: 1.treated and 2.control group must include at least 5 analysable animals per sex and then animals are exposed to the test substance by an appropriate route, after 1, 2, or more treatments at 24 h intervals.

The limit dose has been used, and dosing continued until the time of sampling also be administered as a split dose. It can be given by 2 ways

1) Treated with once. 2) 2 or more daily treatments

1st: Samples of bone marrow =24hr, peripheral blood twice =36hr

2nd: bone marrow samples collected once between 18 and 24 hours, peripheral blood: sampling between 36 and 48 hours.

Bone marrow cells are usually obtained from the femurs or tibias and stained using established methods.

Blood: tail vein or other appropriate blood vessel, smear preparations are made and then stained

DNA specific stain e.g. acridine orange or Hoechst 33258 plus pyronin-Y and at the end observe the presence of micronuclei (Fig.3). (NCBI, 2020; OECD, 1981)

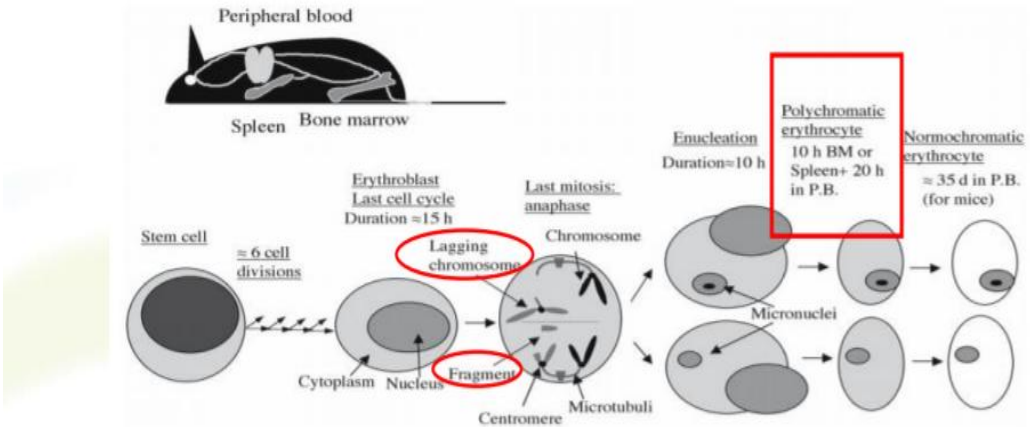

Fig. 3: Mammalian Erythrocytes Micronucleus Test

TG 473: In-Vitro mammalian chromosomal abberation test

Principle: metaphase cells are analysed microscopically for the presence of chromosome aberrations. And identify the agents which can cause structural mutations in chromosomes or chromatids, chromatid mutation being the common. Other type of chromosomal changes like polyploidy and duplication can also be found using this test.

Procedure: Treatment of test with lymphocytes started at about 48 hours after mitogenicstimulation. Cells should exposed to the test substance both with and without metabolic activation for 3-6 hours.sampled at a time equivalent to about 1.5 normal cell cycle length after the beginning of treatment.

Chromosome preparation: culture treated with Colcemid or colchicine 3 hr prior to harvesting, process involves hypotonic treatment of the cells, fixation and staining.

Analysis: should be independently coded before microscopic analysis. At least 200 well-spread metaphases should be scored per concentration and control

it is important to record polyploidy and endoreduplication when these events are seen. And Treatment of results we observe that % of cells with structural chromosome aberration. (OCED, 1981)

In-vivo genotoxicity testing methods: The in-vivo genotoxicity test or assays are done supplemental to in-vitro assay if an in-vitro positive result is obtained. Some of the in-vivo tests done are as follows,

In-vivo comet assay: It is one of the commonly used in-vivo test used for hazard assessment of agents which have potential for genotoxicity or mutagenicity. It helps in detecting the DNA damage and detects a broad variety of primary DNA lesions which cannot be identified by any other tests. This test can be applied to a wide variety of tissues or any special cell types. Being sensitivity to even low level of DNA damage it requires only small amount of cells per sample and it can be completed in a short period of time.

In-vivo micronuclei test/In-vivo chromosome aberration test: It is a test done to identify the damage done chromosome or spindles. On exposure to the mutagen the cell may undergo damage and on division it will form smaller micronucleus additional to the main nucleus. (EMEA, 2006)

Conclusion

Genotoxins are agents that can interact with the DNA thus causing mutations and damaging its structure and may lead to cancer. They act by changing the chromosomal structure by addition, deletion, duplication, forming rings etc. The mutations may lead to a wide variety of diseases to cancer. It is very important to do genotoxicity studies so as to avoid the potential damage that can be caused by it. These genotoxicity tests are done to identify if a drug or other substance have the potential to cause mutation and genotoxicity. By doing so they help us identifying the hazards in the early stage of drug development itself. Identification of the genotoxic agents helps us understand the mechanism of the mutation and genotoxicity thereby paving us way to better prevent the frequency of such mutation and genotoxicity. The development of broad range of short-term assays for genotoxicity serves to identify many mutagens and their relationship with cancer causing agents. Genetic toxicology data is essential for regulatory approval of new chemical entity.

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