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A REVIEW ON GENOTOXICITY, ITS MOLECULAR MECHANISMS, REGULATORY TESTING IN DRUG DEVELOPMENT PROCESS

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ABSTRACT: Genotoxicity has fully-grown into a serious question for the cause of several cancers. In this article, we discuss the basics of genotoxicity, the chemicals which cause these genetic damages and also their mechanism of action. The discovery of new drug needs a thorough investigation for its safety and efficacy before their release into the market so, the drug development process and drug attrition, regulatory tests to detect compounds with genotoxic and carcinogenic potential are discussed in the article. And also, a brief discussion about the recent advances in genotoxicity testing, pre-clinical investigations generally to obtain the basic fundamental profile of toxicological and new chemical entities (NCE), the integration of the cytogenetic tests into repeated dose toxicity studies can be used to satisfy the *in-vivo* cytogenetic data requirement in CFR are explained. Finally, a brief account of the drugs being used in present days, and also some plant products which show antimutagenic effects have been emphasized.

INTRODUCTION: 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 knockout leading to mutation are known as genotoxic.

Further, some classes of substances which are capable of damaging and interacting the genome within a cell are known as genotoxins. Genotoxins include both radiation and chemical genotoxins. Anyway, all genotoxic substances are not mutagenic, and all mutagens are genotoxic.

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Genotoxins can be of the following category depending on its effects [1]:

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*. Genotoxic chemicals which exert their adverse effects through interaction within the cells of genetic material [2]. During the synthesis of drug substances, genotoxic impurities may also generate by involving the use of many chemicals, based on the starting materials, route of synthesis, reagents catalysts, solvents, intermediates and also other processing aids. In another way of genotoxic impurities can be raised due to undesired side reactions between processing materials and degradants. Example reaction between sulfonic acid and alcohol to form sulfonate ester.

During the synthesis of the multistep process of drug substance, *i.e.* active pharmaceutical ingredient, there is a high chance for the formation of impurities along with the AP, *i.e.* active pharmaceutical ingredient. Sources of impurities are process related to the drug substance, starting materials or also known as intermediate impurities, organic or inorganic reagent, enantiomer impurities, catalysts, heavy metals, degradation drug substance, residual solvents and also organic degradation products. The impurities more over alter the properties of certain compounds and bind with the DNA of human being and cause cancer. The residual solvents also induce the physicochemical properties of the drug substances such as crystal nature of the bulk drug, which in turn may concern the solubility properties, odor and color changes in final products.

Hence, these genotoxic impurities are also having a significant effect even in low concentration, which damages the DNA sequence and its structure [3]. However, if any disturbance of genetic is not accurately remodeled, long-lasting lesion apparatus of genetics may appear later replication of cell, this circumstance known as mutagenicity. There are several techniques entrenched and are capable in identifying impairment of genetics also mutations in a large range of end-points, some are DNA strand breaks, point mutations, translocations in chromosomes, chromosomal loss or interference with spindle cell apparatus. Such techniques are identified by international agencies are battery tests recommended for validation of chemical agents that are discharged into the global market. This is very decisive in analyzing risks for human health induced eventuality such new chemicals [4].

Micronucleus test, chromosomal variation test, and many other tests are utilized for contemplating anti-mutagenic action of a medication. Most ideal approaches to limit the impact of mutagens and cancer-causing agents is to recognize the anti-clastogens / anti-mutagens (substances which stifle or hinder the procedure of mutagenesis by acting specifically on the component of cell) and des-mutagens (substances which some way or another annihilate or inactivate, mostly or completely the mutagens, in this way influencing less cell populace) in our eats less and expanding their utilization. Nature has presented us with restorative plants. There is a need to investigate them for use as anti-mutagenic, and anti-carcinogenic nourishment or medication added substances.

In hereditary qualities, genotoxicity portrays the property of concoction operators that harms the hereditary data. The perpetual, inherited changes can influence either physical cells of the life form or germ cells to be passed on to who and what is to come. Cells avert articulation of the genotoxic transformation by either DNA repair or apoptosis; be that as it may, the harm may not generally be settled prompting mutagenesis [5].

Mutations: Transformations which modifies in the DNA succession of cell's genome and are brought about with the aid of radiation, infections, transposons furthermore, mutagenic artificial compounds, and moreover mistakes that appear amid meiosis or DNA replication. There is no accord among hereditary toxicologists with admire to the arrangement of changes.

Three teams of mutations are often distinguished:

1. Single purpose mutations or sequence mutations: These square measures small changes within the deoxyribonucleic acid at the extant of the bases and gens, that square measure invisible underneath a light-weight magnifier. It again includes-

a) Nucleotide substitutions.
b) Addition or deletion of bases.

2. Structural body aberrations.
3. Order mutations.

Anti-mutagen: Anti-mutagen is portrayed as partner specialist that decreases the apparent yield of unconstrained and incited changes. Meanwhile, there are two noteworthy procedures of hostile to mutagenesis-

1. Des-mutagenesis inside which factor on mutagens or inactivate them.

2. Bio anti-mutagenesis in which factors follow up on the procedure of mutagenesis or repair DNA harms that end in an abatement inside the transformation recurrence. Gemcitabine utilized as an operator with an antagonist of metabolites movement applies its effect by precluding deoxyribonucleic corrosive chain stretching [6].

Molecular Mechanisms involved in the Production of Chromosomal Aberrations: 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. Fortunately, cells are equipped with several DNA repair systems. Depending on the specific classes of DNA lesions, one or more DNA repair pathways become active [7]. Four of the 5 major DNA repair pathways are involved in the repair of DNA lesions leading to gene mutations: direct repair, base excision repair (BER), nucleotide excisions repair (NER) and mismatch repair [8]. The 5th major repair pathway involved is single/double-strand break repair.

A. Direct Repair: Direct repair acts by removing or reversing the DNA lesions by a single enzyme reaction in a basic error-free manner and with high substrate specificity. This mechanism does not require a template since the damage they restore only occurs in one base and there is no involvement of incision of the sugar-phosphate backbone or base excision. 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. Next to AGMT, in bacteria and yeast, photolyases can directly reverse UV-induced DNA damage [9, 10, 11].

B. Base Excision Repair (BER): Base excision repair (BER) is a cellular mechanism that repairs damaged DNA throughout the cell cycle. This mechanism protects cells from the deleterious effects of endogenous DNA damage induced by hydrolysis, reactive oxygen species, and other intracellular metabolites, and is also responsible for the removal of many lesions induced by ionizing radiation and strong alkylating agents. The main enzymes involved in BER are DNA glycosylases and AP endonucleases. The DNA glycosylases are involved in the excision of the damaged base, whereafter the remaining a-basic site is further processed by AP endonucleases. BER is divided into short-patch repair (where a single nucleotide is replaced) or long-patch repair (where 2-10 nucleotides are replaced [12, 13].

C. Nucleotide Excision Repair (NER): Nucleotide excision repair (NER) is a repair pathway that is involved in the removal of several kinds of DNA lesions which mainly originate from exogenous sources like UV light or genotoxic chemicals producing bulky adducts and DNA cross-links [14, 15]. NER consists of two different sub-pathways: global genome repair (GGR) and transcription-coupled repair (TCR). These two subpathways are only different in the first step of DNA damage recognition. The first pathway (GGR) eliminates DNA damage present in the genome overall. DNA recognition is accomplished by a complex of protein factors (XPC-HR23B and XPE). The second pathway (TCR) removes lesions from active genes. Hereby, the primary trigger in the DNA damage recognition is a stalled RNA polymerase II, which is accompanied by Cockayne syndrome (CS) proteins [16, 17]. The next stages involved in DNA repair are mostly studied for GGR but are identical in the TCR pathway. After binding of the XPC-HR23B complex to the damaged DNA in GGR, several other proteins are bound such as a complex called transcription factor IIH (TFIIH) and the endonuclease XPG. TFIIH contains two DNA helicase activities with opposite polarity (XPB and XPD) that unwind the DNA duplex. After binding of the replication protein, A (RPA), the damage is verified by XPA, where after the endonucleases XPG and ERCC1/XPF cleave the 3, and the 5, of the DNA lesion. This results in the release of a fragment, containing the DNA damage, of 27-30 nucleotides.

The remaining gap is filled in by a complex formed by DNA polymerase d or e, the accessory replication proteins, the proliferating cell nuclear antigen (PCNA), RPA and the replication factor C [18].

D. Mismatch Repair (MMR): Mismatch repair (MMR) is a system that recognizes and repairs erroneous insertions, and misincorporation of bases. These can arise during DNA replication, and MMR is a strand-specific repair. During DNA synthesis, the newly synthesized (daughter) strand may include incorrect bases. Examples of mismatch bases include base pairs like G/T or A/C. To repair these mismatched base pairs correctly, it is very important to discriminate between the newly synthesized (mismatched) strand and the parental strand.

The first step in MMR is recognition of the deformity caused by the mismatch. After that, the template and the non-template strand are determined, and the incorrect incorporated base is excised and replaced with the correct nucleotide. During the repair process not only, the mismatched nucleotide is removed, but a few or up to thousands of bases of the newly synthesized DNA strand can be removed and replaced [19].

E. Chromosomal Aberrations and Repair: The other endpoint of genotoxicity, chromosomal aberrations, is caused by clastogenic chemicals. Chromosome aberrations can either be structural (clastogenic) or numerical (an-eugenic). DNA damages like double-strand breaks (DSBs) threaten the integrity of chromosomes and viability of cells. Unrepaired or mis repaired DSBs can lead to mutations, chromosome rearrangements, cell death and cancer [20, 21, 22, 23]. Numerical chromosome aberrations (aneuploidy) can be either loss or gain of chromosomes per cell (like trisomy 21 in Down syndrome) and can be lethal or cause genetic diseases. Fortunately, we also possess systems to repair DSBs, the last of the earlier mentioned repair systems. In mammalian cells, DSBs are mainly repaired by either homologous recombination repair (HRR) or nonhomologous end-joining (NHEJ) repair, respectively [24, 25]. The main difference in HRR and NHEJ is the requirement of a homologous DNA sequence in HRR, which is, therefore, an error-free mechanism.

In contrast, NHEJ, which does not use sequence homology is an error-prone mechanism [26, 27]. Another difference is their dependency on the cell cycle. HRR depending on the presence of an intact sister chromatid is more efficient during late S and G2 phase of the cell cycle when sister chromatids are active in dividing cells. NHEJ not depending on a homologous DNA strand can repair DSBs in all cell cycle stages, G1, S, and G2 phase [28, 29, 30, 31]. It has been shown that HRR acts at the embryonic stage, where the embryonic cells were sensitive towards ionizing radiation, but its action in adults was not detected unless NHEJ is disabled. It was concluded that the contribution of HRR and NHEJ could differ depending on the mammalian developmental stage (*i.e.,* cell type) and on the specific type of DNA damage.

F. Homologous Recombination Repair: Homologous recombination repair is an error-free repair system. The RAD52-group of proteins, including RAD50, RAD51, RAD52 and RAD54, and MRE11 play a major role in HRR. In the case of a DSB, the initial cellular response is their cognition of this break through the RAD50/MRE11/NBS1 complex. Subsequently, followed by nucleolytic processing of the broken ends of DNA into 3,- end single-stranded DNA.

The single-stranded DNA is bound by RPA (replication protein A). After RPA is removed and replaced by RAD51, the RAD51 nucleoprotein filament mediates the search for a homologous duplex template DNA where after the complex of joint molecules between the broken DNA ends and the intact ds DNA repair template is formed. The Rad52, Rad54, Rad50 paralogues (such as Rad51B, Rad51C, Rad51D), Xrcc2, Xrcc3 andDmc1 are accessory to Rad51 at various stages of HRR.

After polymerization of nucleotides to restore degraded DNA strands and resolution of the recombination intermediates, the HRR is completed resulting in an error-free double-stranded DNA. The breast-cancer-susceptibility proteins BRCA1 and BRCA2 are involved in HRR as well; however, their role is not well understood [32, 33]. Loss of most HRR factors can lead to early or mid-embryonic lethality in mice [34]. This suggests that HRR plays an important role in development, presumably to repair spontaneously arising DNA damage which is

in agreement with the findings that HRR and NHEJ can play different roles during the mammalian developmental stages [35].

G. Non-Homologous End-Joining Repair: Non-homologous end-joining (NHEJ) is an error-prone repair mechanism. There are at least 3steps involved in NHEJ. The first step is the detection of the strand break and the end-binding mediated by DNA-PK consisting of the three subunits DNA-PKCS (DNA-dependent protein kinase catalytic subunit) and the KU80/KU70 heterodimer, which are involved in the formation of a molecular bridge that holds the broken DNA together. Hereafter, the NBS1/MRE1/RAD50 complex is involved in the processing procedure that modifies nonmatching and damaged DNA ends into incompatible and ligatable ends. Finally, in the ligation step, a complex consisting of DNA ligase IV and XRCC4 (X-ray-repair-cross-complementing defective repair in Chinese hamster mutant ligates the two DNA ends together forming an intact double-strand DNA molecule [36]. Recently, Cernunnos-XLF was discovered, which is also involved in NHEJ61. Cernunnos-XLF interacts and stimulates the DNA ligase IVXRCC4 (LX) complex, which acts in the final ligation step in NHEJ [37].

The Drug Developmental Process and Drug Attrition: The costs spend on R & D have increased tremendously during the last decades. In spite of the increase in R & D expenses, the development of new assay methods, new techniques in liquid handling, robotics, analytical tools and software, the yearly number of approved new drugs has declined [38].

The process of drug development is shown in **Fig. 1** and can be divided into discovery, exploratory development, and the full development and launch of the drug. In the discovery phase, biological targets are validated, and high-throughput screening is used to find molecules that interact with these targets (hits). After identification of the most promising hit (lead molecule), this molecule is further optimized (lead optimization) to a compound that shows pharmacological activity in an animal model. This first phase that ends with delivering a development candidate takes around 3-4 years. The second, exploratory development phase, consists of preclinical development and first into man studies (FIM). *In-vitro* and *in-vivo* testing is performed to assess the safety of the compound. Assays to show the genotoxic potential of compounds is also performed in this phase. When this first set of regulatory assays shows no serious adverse effects an investigational new drug application (IND) is filed to the regulatory authorities such as the Food and Drug Administration (FDA) and European Medicines Agency (EMA). Then FIM phase I clinical studies can be initiated, study the safety and pharmacodynamics of the compound in healthy humans. Successful completion of this second phase results in the acquirement of a statement of no objection (SNOB). The process from development candidate to SNOB takes again around 3-4 years.

In the third phase, large clinical studies (phase II, III) and complex *in-vivo* animal studies like carcinogenicity testing are performed. Results that indicate the absence of adverse effects can lead to the delivery of a full development candidate (FDC) which after a positive review by regulatory authorities will result in the launch of a new prescription drug. This third phase takes around 6-8 years and is the most expensive part of drug development. Thus, the complete process of developing a new drug takes approximately 12-16 years. The average developmental costs of a new prescription drug are high and estimated at approximately 800 million USD [39].

This figure does not take into account the costs of failed drugs as there is a high attrition rate of 90%. When this high attrition rate is taken into account, the costs of developing one new drug can go up to 1.5 billion.

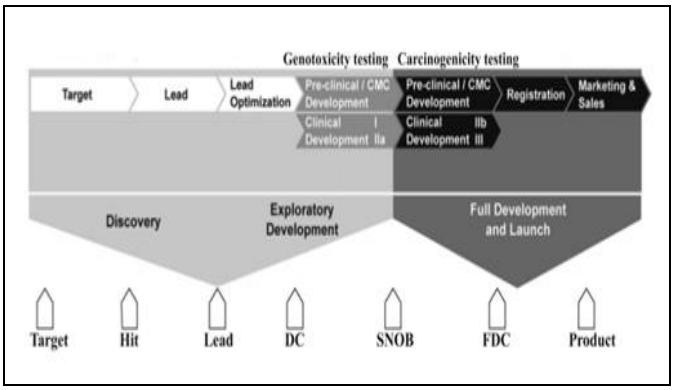

FIG. 1: OVERVIEW OF THE DRUG DEVELOPMENT PROCESS

The development of a new prescription drug takes around 12-16 years. The first phase that takes around 3-4 years to consist of target validation hit/ lead finding and lead optimization and ends with the selection of a development candidate (DC). The second phase consists of preclinical safety studies and the first into man clinical studies. This phase ends after around 3-4 years with a statement of no objection (SNOB). In the third phase that takes around 6-8 years large clinical studies and complex preclinical studies are performed. This phase can deliver a full development candidate (FDC) and after review and registration result in the market launch of a new prescription drug. Genotoxicity and carcinogenicity testing is respectively performed during exploratory and full development.

Genotoxicity Testing: The purpose of genotoxicity testing is to decide whether a substrate will impact genetic material or may cause growth. Which may be executed in different types of cells (i.e., bacterial, yeast, mammalian cells). With the information from the tests, one can control the early advancement of defenseless life forms to genotoxic substances. Genotoxicity tests can be characterized as *in-vitro* and *in-vivo* tests intended to recognize compounds which incite genetic damage directly or indirectly by different mechanisms. This testing of new chemical elements (NCE) is for the most generally utilized for hazard identification concerning DNA harm and its fixation [40]. These damages can appear as a quality transformation, structural chromosomal aberration, recombination and moreover, numerical changes. These movements are responsible for heritable results on germ cells and impose danger to future generations [41]. Also, it has been well documented that somatic mutations can also play an essential role in malignancy [42]. These tests have been utilized for the prediction of cancer-causing nature and genotoxicity because compounds, which are positive in these tests, can be human carcinogens as well as mutagens. Genetic alterations in somatic cells may cause cancer if they occur in genes. Alternatively, they may be responsible for a variety of other non-cancer diseases (genetic diseases). Accumulation of DNA damage in somatic cells has been identified with degenerative conditions for example accelerated aging, immune dysfunction, cardiovascular and neurodegenerative diseases. In germ cells, DNA harm is related with spontaneous premature births, infertility or heritable damage in the offspring and subsequent generations resulting in genetic diseases [43].

A Standard Battery for Genotoxicity Testing: There are two fundamental areas in which harmonization of genotoxicity testing is considered necessary is

a. Identification of a standard set of tests to be conducted for registration.
b. 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.

(a) S2A Guideline: The S2A guidelines cover the strategic issues and protocol design for *in-vitro* and *in-vivo* genotoxicity test [44].

TABLE 1: THE FOLLOWING TESTS ARE THE STANDARD BATTERY TESTS FOR THE EVALUATION OF GENOTOXICITY [45]

Bacterial reverse mutation test (Ames Test)	TG 471
Genetic toxicology: *Escherichia coli*, invert the test	TG 472
In-vitro mammalian chromosome aberration test	TG 473
Mammalian erythrocyte micronucleus test	TG 474
Mammalian bone marrow chromosome aberration test	TG 475
In-vitro mammalian cell gene mutation test	TG 476
Genetic toxicology: Sex-connected recessive lethal test in drosophila melanogaster	TG 477
Genetic toxicology: Rodent dominant lethal test	TG 478
Genetic toxicology: *In-vitro* sister chromatid exchange measure in mammalian cells	TG 479
Genetic toxicology: *Saccharomyces cerevisiae*, gene mutation assay	TG 480

Genetic toxicology: *Saccharomyces cerevisiae*, mitotic recombination assay	TG 481
Genetic toxicology: DNA damage and repair, unscheduled DNA synthesis in mammalian cells *in-vitro*	TG 482
Mammalian spermatogonial chromosome aberration test	TG 483
Genetic toxicology: Mouse spot test	TG 484
Genetic toxicology: mouse heritable translocation assay	TG 485
Unscheduled DNA Synthesis (UDS) test with mouse liver cells *in-vitro*	TG 486
In-vitro mammalian cell micronucleus test	TG 487

Regulatory Tests to Detect Compounds with Genotoxic and Carcinogenic Potential: Regulatory tests to detect genotoxic potential a stepwise approach (tiered approach) is applied in regulatory genotoxicity testing [46]. *In-vitro* assays with high sensitivity are used as a first step to see whether the test compounds have intrinsic genotoxic activity. These tests are then followed by *in-vivo* tests that are designed to assess the relevance of the *in-vitro* result for the *in-vivo* situation. *In-vivo* genotoxicity studies are also performed for the reason that some genotoxicants are only detected *in-vivo* [10]. A decision tree for regulatory genotoxicity testing including a description of necessarily follow up testing is described in detail later in this chapter. Genotoxicity testing is in comparison to the regulatory testing of carcinogenicity relatively cheap and fast. Compounds without genotoxic liability can proceed to FIM clinical trials. The carcinogenic potential is further assessed in the full developmental phase of drug development. The regulatory test strategy consists of a battery of tests because the three types of genotoxicity (gene mutations, clastogenicity, and aneugenicity) cannot be detected by a single test. The standard test battery required for genotoxicity testing is described in ICH guideline S2B for the registration of pharmaceuticals for human use and consists of

(1) The Ames assay to detect gene mutations in bacteria, (2) an *in-vitro* chromosome aberration or mouse lymphoma TK assay in mammalian cells and (3) an *in-vivo* chromosome damage assay (chromosome aberration or micronucleus assay). Specific technical aspects of these regulatory tests are described in ICH guideline S2A for the registration of pharmaceuticals for human use. The assays from the standard test battery have different sensitivity, specificity, and predictivity for carcinogenicity. For the calculations of the sensitivity, the results of the genotoxicity assays are compared with the results of the carcinogenicity tests. It is, however, important to note that several of the carcinogenic compounds act via a non-genotoxic mode of action. Genotoxicity tests will thus never reach a sensitivity of 100% for carcinogenicity. The assays from the regulatory genotoxicity test battery are described in more detail in the next sections and the performances scores are shown in **Table 2**.

TABLE 2: PERFORMANCE DEFINITIONS FOR GENOTOXICITY TESTS

Term	Definition
Sensitivity	Percentage of carcinogens positive in the test
Specificity	Percentage of non-carcinogens negative in the test
Predictivity	Percentage of all tested compound that was predicted correctly

The Ames Assay: The Ames test is an assay that was developed by Bruce Ames and is performed to assess the mutagenic potential of chemical compounds [47, 48]. The assay is performed in Salmonella typhimurium bacteria that carry mutations in genes involved in histidine biosynthesis. As a consequence, the bacterial cells require histidine for growth and are so-called histidine auxotroph's. Mutagenic compounds can cause a reverse mutation which results in bacteria that can grow on a histidine-deficient medium. The number of bacteria that form colonies is then used as a measure for the mutagenic potential of a compound. Several bacterial strains are used that have frameshift or point mutations in the genes required for histidine synthesis.

These diverse strains are used to be able to detect mutagens acting via different mechanisms. Besides the mutations in the histidine synthesizing genes, the tester strains also have additional mutations to make the strains more sensitive for the detection of mutations. A mutation in the genes used for lipopolysaccharide synthesis causes the cell wall of the *Salmonella typhimurium* bacteria more permeable. Moreover, the strains have a mutation in their excision repair system [49]. The specificity of the Ames assay is relatively high in comparison to

the other *in-vitro* genotoxicity tests **Table 2**. The sensitivity, specificity, and predictivity of the Ames assay calculated by Kirkland *et al.*, was 58.8%, 73.9%, and 62.5%, respectively [50]. To mimic metabolism in bacterial (and mammalian) mutagenicity assays, a liver fraction (S9 mixture) containing phase I and II drug metabolizing enzymes from Aroclor 1254 treated male Sprague-Dawley rats are used. Aroclor 1254 stimulates the AhR, pregnane X receptor (PXR) and CAR and leads to high levels of cytochrome P450 (CYP) 1A1, CYP1A2, CYP2B, and CYP3A, which are involved in the activation of a large number of proximate genotoxicants. Assays are performed in the presence and absence of S9 mixture to study whether compounds are activated or inactivated by metabolism.

TABLE 3: THE SENSITIVITY, SPECIFICITY, AND PREDICTIVITY OF THE ASSAYS OF THE STANDARD REGULATORY TEST BATTERY FOR THE ASSESSMENT OF GENOTOXIC POTENTIAL [51, 52]

Assay	Sensitivity (%)	Specificity (%)	Predictivity (%)
Ames	58.8	73.9	62.5
Chromosome aberration (CA)	65.6	44.9	59.8
Mouse lymphoma TK (MLA)	73.1	39.0	62.9
Micronucleus *in-vitro*	78.7	30.8	67.8
Micronucleus *in-vivo*	40.0	75.0	48.0

The Chromosome Aberration Assay: The chromosome aberration assay (CA) is performed *in-vitro* in cultured mammalian cells. Structural and numerical damage is scored by microscopic examination of chromosomes in mitotic metaphase cells. Tests are carried out with and without S9 mixture [52, 53]. This assay is often performed in Chinese hamster ovary k1 (CHO-k1) or lung cells (V79) or human lymphocytes. Scoring needs specialized training and experience. The sensitivity and predictivity of this test are 65.6%, and 59.8% respectively. The specificity of this test is low with only 44.9% [50].

The Mouse Lymphoma TK Assay: Thymidine monophosphate (TMP) is one of the four deoxyribonucleotide monophosphates, TMP does not undergo significant conversion to other nucleotides. This conservation makes the TMP pool size quite small and constant under normal growth condition. Therefore, the TMP pool serves as a regulator for DNA synthesis.

If TMP is replaced by a lethal TMP analog, cells will die. The phosphorylation of these analogs is mediated by the "salvage" enzyme thymidine kinase (TK), which phosphorylates thymidine into TMP in mammalian cells. TK-deficient cells lack this enzyme activity and therefore are resistant to the cytotoxic effect of the lethal analog. In the mouse lymphoma TK assay, the TK-competent L5178Y (TK+/+ or TK+/-) cells are treated with the test agents. After treatment, the cells are shifted to a selective medium containing a lethal TMP analog such as trifluoro thymidine (TFT). Normally most cells will die, however in the presence of a mutagenic compound, TK -/- cells might have been formed which are resistant to the cytotoxicity. The number of cell colonies on test plates is, therefore, a measure for genotoxicity. The size of the colonies gives information about chromosome damage as large changes in the DNA inhibit growth and result in small colonies, whereas large colonies denote gene mutation.

The sensitivity and predictivity of the mouse lymphoma TK assay (MLA) are 73.1% and 62.9%. Similar to the chromosome aberration assay, the specificity of this assay is low with only 39.0% [50].

The Micronucleus Assay: The fourth regulatory genotoxicity assay is the micronucleus assay. Chromosomal fragments or complete chromosomes that are the result of DNA damage or errors in the separation of chromosomes during the cell cycle can sometimes be found outside the nucleus in one of the daughter cells. After the division of the nucleus these DNA fragments will decompensate and form a so-called micronucleus. By using DNA staining techniques, these micronuclei become visible and countable under the microscope. The number of these micronuclei per 1,000 (bi-nucleated) cells is used as a measure for genotoxicity. This assay can be performed *in-vitro* on cell lines like CHO-k1.

Micronuclei can also be measured in red blood cells and bone marrow obtained from *in-vivo* experiments. By using centromeric probes, it is possible to determine whether micronuclei contain complete chromosomes or fragments of

chromosomes. These results can then be used to determine whether compounds have a clastogenic or aneugenic mode of action [54].

The sensitivity, specificity, and predictivity of the *in-vitro* micronucleus assay are 78.7%, 30.8%, and 67.8%, respectively. The specificity of the *in-vivo* micronucleus assay in bone marrow is much higher with 75%. The sensitivity of the *in-vivo* test is lower with 40%, and the predictivity is 48% [50, 51].

The Impact of Positive Findings for Genotoxic Potential and Follow up Testing Strategies: A decision tree for the tiered approach in regulatory genotoxicity testing is shown in **Fig. 2**. In general, a combination of the Ames + MLA + (or) CA is used in in-vitro regulatory testing for genotoxic potential. A combination of the Ames + MLA + CA has a high sensitivity (84.7%) but low specificity (22.9%) for carcinogenicity. When these tests show no genotoxic potential, the *in-vivo* micronucleus test is performed. This *in-vivo* test is performed as there are several compounds that are poorly detected *in-vitro*. For example, proximate carcinogens that are activated by phase II enzymes [55, 56].

When the *in-vivo* micronucleus assay also shows a negative result, it is likely that the compound has no genotoxic potential and the compound can proceed in development. More research is needed in a rare situation, where the *in-vivo* micronucleus assay gives a positive result after negative results *in-vitro*. It has been shown that compounds that increase or decrease the core body temperature for a sustained period, compounds that increase the erythropoiesis in the bone marrow, and compounds that inhibit protein synthesis induce the number of micronuclei in bone marrow *in-vivo*. Experiments to show these modes of action have been described by an IWGT working group [57]. Such positive results are mostly irrelevant for humans. Mechanistic data to demonstrate lack of clinical relevance for humans or a non-DNA reactive mechanism can lead toa continuation of further development. For non-DNA reactive genotoxicants (*e.g.,* topoisomerase inhibitors and spindle poisons) a threshold might be justified. In case of a DNA reactive mode of action, development is terminated. In the case of a positive result in the *in-vitro* genotoxicity assays, it is required to perform at least two follow up *in-vivo* genotoxicity tests. These are the *in-vivo* micronucleus assay and another test. In the past, the UDS tests was often used. But nowadays the Comet assay is more preferred in the testing for human pharmaceuticals [58]. This because it has been shown that most *in-vivo* micronucleus negative carcinogens giving DNA adducts are detected in the Comet assay. Of these compounds the tiered approach is highly sensitive. About 80%-90% of the carcinogens are detected; however, the specificity of especially the *in-vitro* mammalian genotoxicity assays is very low [59]. This is also supported by the retrospective analysis performed by Snyder and Green [60]. They showed that 50% of non-carcinogenic marketed drugs have a positive result in the mammalian genotoxicity assays, indicating the high false positive rate of these tests.

Two negative results *in-vivo* overrule in principle a positive result *in-vitro*, however in the case of development of pharmaceuticals for human use often additional investigations are performed to get a clue about the reason for the positive result. In the case, the *in-vivo* tests are additional positive investigations might also be useful to show whether the positive *in-vivo* result is relevant for humans or that the compound acts by a threshold mode of action. In this way, the compound can be saved from attrition. A summary of human non-relevant, indirect or threshold mechanisms of genotoxicity is given in **Table 4.**

In a paper from Kirkland *et al*., [61] *in-vitro* approaches are described to determine whether these effects occur in or are relevant for humans. The difficulty, however, is to predict what mechanism is affected by a compound giving a positive *in-vitro* or *in-vivo* genotoxicity result. Toxicogenomic approaches might be very valuable in this aspect as they can give a clue about the mechanism of action [62].

In general, the following test strategy is used to assess the mode of action after a positive result *in-vitro* that is suspected to be not relevant for the human situation or suspected to have a threshold [61]. Firstly, *in-vitro* assays are performed to show the indirect or threshold mode of action. Then *in-vivo* tests are performed. When these tests are positive evidence must be obtained that this positive result

is caused by the same mode of action. In case of a human-relevant non-DNA or threshold mode of action, the NOAEL (No Observed Adverse Effect Level) must be determined. When the anticipated human dose is much lower development of the compound might continue. In case of a for human-relevant DNA reactive mode of action development of the compound is terminated.

TABLE 4: SUMMARY OF HUMAN NON-RELEVANT, INDIRECT OR THRESHOLD MECHANISM OF GENOTOXICITY. THE *IN-VITRO* SYSTEMS AFFECTED AND THE PROBABILITY TO OBTAIN EXPERIMENTAL EVIDENCE TO SUPPORT THE MECHANISM ARE SHOWN [61]

Mode of action	Description	*In-vitro* system affected	Possibility to obtain experimental evidence
In-vitro specific	Rat S9 mixture specific effects	All, except primary hepatocytes	Reasonable
	Feeding effects	Bacteria	Reasonable
Direct DNA effect but with a threshold	Azo- and nitro- reduction	Bacteria	Reasonable
	DNA repair deficiency	All	Difficult
	Inadequate detoxification	All	Reasonable
	Metabolic overload (production of reactive oxygen species, lipid peroxidation, and sulphydryl depletion)	Mammalian cells	Reasonable
Indirect effect	Inhibition of topoisomerases	Mammalian cells	Reasonable
	Inhibition of kinases	Mammalian cells	Reasonable
	Inhibition of DNA polymerases	Mammalian cells	Reasonable
	Imbalance of DNA precursors	Mammalian cells	Reasonable
	Energy depletion	Mammalian cells	Difficult
	Inhibition of protein synthesis	Mammalian cells	Difficult
	Nuclease release from lysosomes	Mammalian cells	Difficult
	Protein denaturation		Difficult
	Aneuploidy		Reasonable
	High toxicity		Reasonable

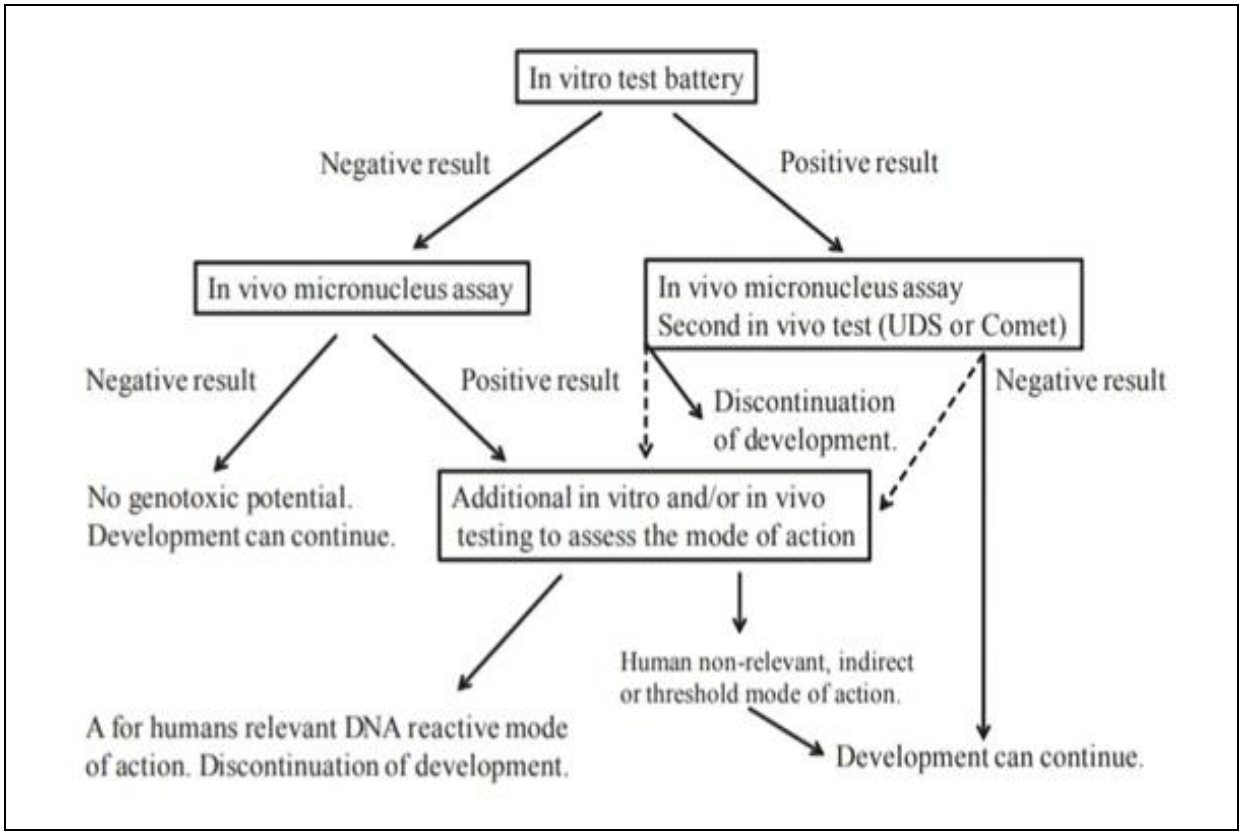

FIG. 2: SCHEMATIC OVERVIEW OF THE DECISION TREE AND NECESSARY, FOLLOW UP TESTING FOR REGULATORY GENOTOXICITY TESTING IN CASE OF PHARMACEUTICALS FOR HUMAN APPLICATION

New Methods in Genotoxicity Testing: In general, the three standard genotoxicity test battery is adequate for evaluation of genotoxicity of an NCE. However, on rare occasions, the standard battery may be inadequate, necessitating further testing. Such additional testing may provide mechanistic information for chronic rodent carcinogenicity bioassay. The ICH guidelines do not exclude the new methods and encourage the development of new systems and their use when strong scientific justifications support the findings. Many mutagens physically form adducts with DNA either directly or after metabolic activation. So highly sensitive and specific analytical methods like 32P-post-labeling immunological assays using polyclonal and monoclonal antisera and mass spectrometry are employed for adduct analysis. For the detection of single and double DNA strand breaks, 'the comet assay' (single cell gel electrophoresis or SCGE) provides a rapid visual method for quantitative estimation. The transgenic mice model, which provides an opportunity to study *in-vivo* gene mutation and to understand the complex mechanism of carcinogenesis has a greater potential for genotoxicity testing. Other tests like an assessment of p53 gene mutation identification of apoptosis detection of aneuploidy by anticentromere antibody use of fluorescent *in situ* hybridization (FISH) to visualize translocation of chromosomes detection of unscheduled DNA synthesis (UDS) and cell transformation assay can be used for genotoxicity screening. These tests will increase both the sensitivity and specificity of the existing test protocols [63].

Current Genotoxicity Testing in Preclinical and Drug Development Process: Genotoxicity testing offers a simple, relatively inexpensive way to identify compounds that may interact with DNA or cause chromosome loss. These tests are required by regulatory agencies for small molecules before clinical trials and for impurity qualifications. Pharmaceutical companies often use these tests, modified versions, or other indicator tests throughout the drug discovery and development process to eliminate potentially genotoxic chemical series, to ensure that development candidates have insignificant genotoxic liability and to clarify the risk to human health after a positive finding in one or more assay. Individual genotoxicity tests identify hazards; a weight of evidence approach can be used as part of a comprehensive risk assessment. While most routinely used genetic toxicity tests are decades old, the discipline will most likely benefit from additional mechanistic clarity that novel technology, like toxicogenomics, can provide the information. Genotoxicity and mutagenicity examinations have a huge role in the distinguishing of hazard impacts of therapeutic drugs, cosmetics, agrochemicals, industrial compounds, food additives, natural toxins and nanomaterials for regulatory purposes [64]. To assess mutagenicity or genotoxicity, distinctive *in-vitro* and *in-vivo* techniques exert various geno-toxicological endpoints such as point mutations, changes in number and structure of chromosomes. The limitations that have arisen as a result of the regular utilization of some of the methods. The solution of actual and practical problems of genetic toxicology is inarguably based on the understanding of DNA damage mechanisms at molecular, subcellular, cellular, organ, system and organism levels. Current strategies to investigate human health risks should be modified to increase their performance for more reliable results and also new techniques such as toxicogenomics, epigenomics, and single cell approaches must be integrated into genetic safety evolutions. The explored new biomarkers by the omic techniques will provide forceful genotoxicity assessment to reduce the cancer risk [65].

Combining Genotoxicity Testing with Standard Repeated Dose Toxicology Testing: The integration of the cytogenetic tests into repeated dose toxicity studies can be used to satisfy the *in-vivo* cytogenetic data requirement in CFR Part 158 and Part 161. The evaluation of micronuclei in peripheral blood or bone marrow cells covers the evaluation of structural and numerical chromosomal aberrations. The integration of the mammalian bone marrow and the rodent erythrocyte micronucleus assays is technically feasible and is a scientifically acceptable alternative to conducting independent *in-vivo* cytogenetic assays. The integration of a cytogenetic assay into repeat-dose toxicology studies is internationally accepted, as discussed in papers by the International Workshop on Genotoxicity Testing [66] and the International Committee for Harmonization (ICH) for pharmaceuticals

Guidance for conducting a cytogenetic test that is integrated into a repeat-dose toxicity study can be found in the ICH guidance [67]. Additional guidance on the evaluation of micronuclei (MN) can be found in other harmonized test guidelines (*e.g.*, OECD test guideline 474[1]). Some general guidance is provided below:

a. Rats from repeat-dose studies (7- 90 days) bled for toxicokinetic investigations or other routine toxicological purposes can be used for MN analysis, or a small blood sample may be obtained specifically for the MN analysis. When studies of 28-90 days' duration in mice are conducted, there is an additional advantage in obtaining the MN frequency in mature red blood cells at steady state (*i.e.*, after approximately 1 month of exposure) to obtain a measure of the average extent of damage over the one month before sampling [68]. The MN frequency in reticulocytes provides a measure of damage occurring approximately 2 days before sampling.

b. The doses tested are generally considered appropriate when the toxicology study meets the criteria for an adequate study. Further guidance can be found in ICH and OECD test guidelines (474). Routine MN determination is at terminal sacrifice (*i.e.*, day after the final administration) and should be sufficient in most cases, although it has been recommended that an additional early sampling at approximately 3-4 days is advantageous [69].

c. The number of animals analyzed is determined by current test guideline recommendations for the micronucleus assay (OECD TG 474) and generally does not include all the animals treated in a guideline toxicology study. Animals used for MN analyses should be randomly selected from the group used for the toxicology study.

d. Samples for MN analysis can be collected from both sexes, but single-sex can be scored if there is no substantial sex difference evident in toxicity/metabolism.

e. Route of administration is generally the route used in the repeat-dose toxicity study, *e.g.*, oral (feeding or gavage), but can be modified if appropriate to obtain systemic exposure, *e.g.*, for topically applied compounds.

f. It is considered sufficient to treat animals with a positive control only periodically and not concurrently with every assay after a laboratory has established competence in the use of the assay. Blinded, randomized scoring controls (standards or samples obtained from separate studies and then coded) should be included in each study.

g. Guidance on the evaluation of test results does not differ from the evaluation of independent *in-vivo* cytogenetic assays.

Flow cytometric methods may be used for the enumeration of micronuclei. This method provides more rapid and improved measurements and enhanced statistical power given that more cells can be analyzed and instrument calibrations standards are available. MN results scored by flow cytometric methods are highly correlated with traditional microscopy and provide substantially improved precision [70, 71].

When using any new or amended test protocols, the registrant is encouraged to consult or submit the proposed test protocol to the EPA for review before conducting the study.

Drawbacks of Current Genotoxicity Tests: Majority of the currently used genotoxicity assays for regulatory toxicity testing were developed in the 1970,s. Thus, their throughput cannot meet the requirements of the drug discovery requirements. In most of the cases, the site and mechanism by which the compound produces genotoxicity under the study are not known. It may happen that the target site in the test system may not be the same target site of toxic action of the NCE.

In sub-chronic and chronic toxicity testing, several relevant parameters or endpoints can be detected to determine the toxicity, but the same is rarely true for genotoxicity tests. A single test system cannot be designed for universal detection of all the relevant genotoxic substances. Testing requirements depend upon the nature and category of chemical substances. There is no validated test system for detecting induced genome mutation (aneuploidy) in germ cells.

Prevention of Genotoxicity: Genotoxic effects such as deletions, breaks, and rearrangements can lead to cancer if the damage does not immediately lead to cell death. Regions sensitive to breakage, called fragile sites, may result from genotoxic agents (such as pesticides).

Some chemicals can induce fragile sites in regions of the chromosome where oncogenes are present which could lead to carcinogenic effects. In keeping with this finding, occupational exposure to some mixtures of pesticides are positively correlated with increased genotoxic damage in the exposed individuals [72]. The DNA damage is not uniform in its severity across populations because Individuals vary in their ability to activate or detoxify genotoxic substances, which leads to variability in the incidence of cancer among individuals. The difference in the ability to detoxify certain compounds is due to individuals' inherited polymorphisms of genes involved in the metabolism of the chemical. Differences may also be attributed to individual variation in the efficiency of DNA repair mechanisms [73]. The metabolism of some chemicals results in the production of reactive oxygen species which is a possible mechanism of genotoxicity. This is seen in the metabolism of arsenic which produces hydroxyl radicals, which are known to cause genotoxic effects [74].

Similarly, ROS have been implicated in genotoxicity caused by particles and fibers. Genotoxicity of non-fibrous and fibrous particles is characterized by high production of ROS from inflammatory cells [75]. Flavonoids have been reported to possess a wide range of biochemical and pharmacological activities, both potentially detrimental and protective. One of the effects of flavonoids is the ability to modulate the xenobiotic metabolism. Various studies have indicated that a potential basis for protection is interference with enzymes such as cytochrome p450 which plays an important role in the metabolic activation of a wide range of carcinogens [76].

Drugs presently being used as anti-mutagenic agents are busulfan, carmustine, etoposide, *etc*. Plant-derived polyphenolics and other chemicals with antioxidant properties have been reported to inhibit the expression of genotoxic activity by pro-oxidant chemicals [77]. *In-vitro* and *in-vivo* studies with ionizing radiation suggest that hydroquinone (HQ) may have similar protective effects.

The protective effect of HQ may be due to enzyme induction or a direct antioxidant effect of HQ against oxidants commonly present in the diet [78]. Ellagic acid peracetate (EAPA), which unlike ellagic acid (EA) has demonstrated time-dependent inhibition of liver microsomes catalyzed AFB1-epoxidation as measured by AFB1 binding to DNA. EAPA was more potent than EA in preventing bone marrow and lung cells from AFB1- induced genotoxicity. EAPA was acted upon by microsomal acetoxy drug: protein transacetylase (TAase) leading to modulation of the catalytic activity of specific functional proteins (cytochrome P450, NADPH cytochrome c reductase, and glutathione S-transferase), possibly by way of protein acetylation. Non-flavonoid compounds such as simple phenolics (C6), phenolic acids (C6-C1), cinnamic acid and related compounds (C6-C3) also showed antimutagenic effects[79].

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 too cancer. Nowadays drug discovery and development is rapid, time-saving and productive due to the use of newer technologies. It is essential to do genotoxicity studies to avoid the potential damage that can be caused by it.

These genotoxicity testing are done to identify if a drug or other substance has the potential to cause mutation and genotoxicity. There are many standard battery tests for determining the hazards in the early stage of drug development itself. Recent advances in *in-vivo* and *in-vitro* genotoxicity testing will provide practical consequences in the risk assessment processes and further development of substances. Identification of the genotoxic agents helps us understand the mechanism of the mutation and genotoxicity thereby paving our way to prevent the frequency of such mutation and genotoxicity better.

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