GENOTOXICITY TESTING, A REGULATORY REQUIREMENT FOR DRUG DISCOVERY AND DEVELOPMENT: IMPACT OF ICH GUIDELINES

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ABSTRACT
Regulatory authorities all over the world require data on the genotoxic potential of new drugs, as part of the safety evaluation process. The pre-clinical studies are generally conducted to obtain the basic toxicological profile of new chemical entities (NCE). The toxicological data are used to evaluate the safety and efficacy of NCE, which will help in predicting the drug's likely risk/benefit assessment in New Drug Application (NDA) process. Genotoxicity assays have become an integral component of regulatory requirements. The aim of this review is to discuss the present status of genotoxicity testing for pharmaceuticals, the regional variations in testing procedures and the role of International Conference on Harmonization (ICH) in the adaptation of uniform guidelines. The limitations of different test systems and the integration of new test methods have been discussed. These new guidelines will improve the accuracy of genotoxicity testing, minimize the time requirement for such testing and help to conserve the resources in the process of drug discovery and development.

KEYWORDS
Mutagenicity drug safety preclinical toxicity tests carcinogenicity genotoxic carcinogens

1. Introduction
Genotoxicity testing of new chemical entities (NCE) is generally used for hazard identification with respect to DNA damage and its fixation. These damages can be manifested in the form of gene mutation, structural chromosomal aberration, recombination and numerical changes. These changes are responsible for heritable effects on germ cells and impose risks to future generations. In addition it has been well documented that somatic mutations can also play an important role in malignancy. These tests have been used mainly for the prediction of carcinogenicity and genotoxicity because compounds, which are positive in these tests, have the potential to be human carcinogens and/or mutagens.

1.1. Shift in drug discovery paradigm:
The discovery of new drugs needs a thorough investigation for its safety and efficacy before their release into the market. The financial investment in a new drug discovery grows exponentially as the compound progresses from initial discovery, through development to registration. The old paradigm of drug discovery process has transformed due to the innovation in technology. A large number of molecules with great diversity can be rapidly synthesized by combinatorial chemistry and high throughput screening has enormously increased the scope and speed of biological assays for safety evaluation. The primary objective of drug safety evaluation is to obtain biological information indicative of toxicity, which can be interpreted and/or extended to the assessment of health risk to the humans. The potential risks and benefits of the drug under study are carefully considered, so that the benefits of using a new drug as a therapeutic agent outweigh the risks and side effects produced by it. New drug discovery is a complex process and a NCE moves through different phases (Figure 1) of development either sequentially or concurrently and finally reaches the therapeutic...
Figure 1. Schematic representation of pre-clinical safety assessment.

<table>
<thead>
<tr>
<th>New drug substance</th>
<th>Pre IND</th>
<th>IND</th>
<th>Phase 1</th>
<th>Phase 2</th>
<th>Phase 3</th>
<th>New drug application</th>
</tr>
</thead>
</table>

IND-Investigational New Drug

TOXICITY STUDIES

target. Toxicity assessment of a new molecule can be performed in parallel with the pharmacological assessment early in the evaluation process. Toxicity information provided early in the developmental stages can significantly reduce financial expenditure through avoidance of unnecessary activities and improves efficiency. Preclinical studies are the prime source of information regarding a substance’s biological effects. These results influence not only the decision whether to expose human subjects to NCE but also the design of their clinical trials. The detection of serious biological adverse effects may result in the cancellation of the development of the drug or a return to a basic chemistry to modify the structure and to minimize the risk.

2. Role of National and International regulatory authorities in genotoxicity testing

Most of the countries are having the specific guidelines for testing of pharmaceuticals for genotoxicity. In India, Schedule ‘Y’ of Drugs and Cosmetic Rules 1988, Central Drugs Standard Control Organization (CDSCO), Directorate General of Health Services (DGHS), New Drugs Division issued by Ministry of Health and Family Welfare, Govt. of India, deals with the prerequisites to carry out the clinical trials of a new drug before its marketing, depending upon the status of the drugs in other countries. As per the regulatory requirements, the mutagenicity and carcinogenicity testing are required, when the compound or its metabolite is structurally related to a known carcinogen or when the nature and action of the drugs suggest a mutagenic/carcinogenic potential. In United States the FDA’s (Food and Drug Administration) Centre for Drugs and Biologics Evaluation and Research (CDER and CBER) recommend genotoxicity testing for all new drugs. At present, the FDA accepts the three-test package as required by the Ministry of Health and Welfare (MHW) in Japan. In European Community (EC) mutagenicity data are required for the pharmaceuticals before the commencement of clinical trials and the marketing authorization. However, it has further suggested that additional test might be required in specific circumstances. In Japan, the Ministry of Health and Welfare (MHW) adopted mutagenicity tests in 1984 as one of the several toxicity studies required for the approval to manufacture or import of new drugs. The guidelines include three representative tests: a reverse mutation assay in bacteria, chromosomal aberrations test with mammalian cells in culture and a rodent micronucleus test. These were amended in 1989 with several revisions. The Canadian guidelines consider pharmaceuticals as the greatest level of initial concern for preclinical toxicity testing due to high and wide spread use for humans. The Health Protection Branch (HPB) of the department of health and welfare in Canada requires genotoxicity testing of all drugs based upon the level of concern strategy. The Organization for Economic Cooperation and Development (OECD) guidelines are specifically important, because by complying the same one can ensure the acceptance of toxicity data in each of the thirty member countries of the OECD. The International Conference on Harmonization (ICH) brings together the regulatory authorities of
Europe, Japan and the USA. When the ICH guidelines have been developed the Organization for Economic Cooperation and Development (OECD) updated several of their genotoxicity test guidelines. In fact, both ICH and OECD influenced each other resulting in a similar recommendation. However, due to the specific nature of pharmaceuticals, the ICH guidelines contain pharmaceutical related recommendations, which significantly differ from the recent OECD guidelines.

2.1. Limitations of the present regulatory system to test genotoxicity

Today, it is not possible to register a new drug without providing information regarding its mutagenicity. The current differences in protocol design and practices between different regulatory authorities hinder the drug discovery process and delay the marketing of the potential candidates (Table 1). As per the regulatory requirements to introduce a NCE as drug, the preclinical safety study has to be performed in each country as per that country's guidelines. These are very time consuming, resource intensive processes and need a large number of animals for experimentation. In most of the countries, the guidelines are inadequate to draw a definitive conclusion for the genotoxic potential of a NCE. The in vitro and in vivo protocols mentioned in the guidelines are not validated for detection of genome mutations (aneuploidy) and somatic point mutations. For certain categories of pharmaceuticals, which need critical experimental evaluation, there are no details with regard to the choice of specific test system and test protocols. Most guidelines are devoid of recommendations for compounds, which are genotoxic, but seems to act by non-DNA targets. There are also no specific recommendations on the threshold of different genotoxic and tumorigenic compounds and their organ-specific effects when they are intended to use therapeutically.

3. Genotoxicity testing procedures used in regulatory toxicity

Over the past 15 years, various countries have issued several guidelines on the genotoxicity testing of new drugs. Although no specific tests are prescribed in some countries, it is apparent that most of the guidelines recommended by various regulatory authorities are represented in the four-test battery. These tests include: a gene mutation in bacteria, a test for chromosome aberrations in mammalian cells in vitro; a test for gene mutation in eukaryotic cells in vitro; an in vivo test for genetic damage. The compound under study complies genotoxicity testing, when the results of all the tests in the four-test battery are uniformly negative. Further experiments are suggested when the test results are not uniform in the four-test battery.

In India the eighth amendment of Drug and Cosmetic Rules recommends mutagenicity and carcinogenicity testing under Schedule 'Y' before clinical trials for import and manufacture of new drug substance. No specific test has been recommended for this purpose. However, it is required that at least three dose level mutagenicity test has to be carried out, when the drug or its metabolite is related to a known carcinogen. Recently Government of India constituted a committee consisting of experts to re-evaluate the guidelines. The main objective of the committee is to advise the Drug Controller General of India in matters relating regulatory provisions under Schedule 'Y' of the Drugs and Cosmetics Rules any modifications required thereof. The committee will also undertake in-depth evaluation of data furnished by the applicant for approval of Phase I clinical trial for an Investigational New Drug (IND) or New Chemical Entity (NCE).

The schedule of genotoxicity testing in relation to clinical trials is quite different in different regions. Generally the in vitro mutation test and chromosomal aberration tests are completed before the first human exposure and the standard battery should be completed before the initiation of phase II clinical trials. With the implementation of different guidelines by the regulatory authorities of different countries, follow up of Good Laboratory Practice (GLP) is mandatory to carry out genotoxicity. With this, more reproducible and reliable results can be expected. However, in majority of the cases, inconclusive genotoxic activity was obtained due to inadequate testing. The range and type of genotoxicity studies routinely performed for pharmaceuticals are not applicable to the active components of biotechnology-derived
Table 1. Protocol variations for genotoxicity testing *in vitro* and *in vivo* according to various regulatory guidelines.

<table>
<thead>
<tr>
<th>India</th>
<th>United States</th>
<th>European Community</th>
<th>Japan</th>
<th>Canada</th>
<th>ICH</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Ministry of Health &amp; Family Welfare)</td>
<td>(FDA’s Centre for Drug Evaluation &amp; Research)</td>
<td>(The Commission of the European Union)</td>
<td>(Ministry of Health and Welfare)</td>
<td>(Health Protection Branch)</td>
<td>(Regulatory authorities of EU, Japan &amp; USA)</td>
</tr>
</tbody>
</table>

1. If the drug or its metabolite is related to a known carcinogen.
   - 1. Microbial mutagenicity test.
   - 1. Bacterial reversion test.
   - 1. Salmonella/microsome assay.
   - 1. A test for gene mutation in bacteria.

2. Two species should be used for carcinogenicity studies.
   - 2. *In vitro* mammalian cell mutagenicity test.
   - 2. *In vitro* chromosomal aberration test.
   - 2. *In vitro* chromosomal damage with mammalian cells or an *in vitro* tk assay.

3. At least 3-dose level should be used.
   - 3. Mammalian chromosome test *in vitro*.
   - 3. *In vivo* micronucleus test.
   - 3. Mammalian *in vivo* assay (either metaphase or micronucleus test).
   - 3. *In vitro* test for chromosomal damage using rodent haemopoietic cells.

4. A control group should always be included.
   - 4. *In vitro* mammalian cell transformation assay.
   - 4. An appropriate *in vivo* assay (usually test for chromosomal aberration).
   - 4. *In vivo* micronucleus test.
   - 4. Positive *in vivo* results may need additional *in vivo* germ cell assay.
   - Additional tests:
     - (i) Continuous treatment for 24 and 48 hours with and without S9 mix.
     - (ii) Pulse treatment for 6 hours (with and without S9 mix) followed by sampling at 24 hours.
     - (iii) Chromosome preparation for the presence of polyploid cells
     - (iv) Use of single sex (male) in rodent micronucleus test
     - 2. DNA-strand breaks.

5. Cytogenetic tests *in vivo* (e.g. bone marrow micronucleus test, liver unscheduled DNA synthesis [UDS] test).

6. Further *in vivo* test selection is left to the applicant
   - Additional tests: (i) Continuous treatment for 24 and 48 hours with and without S9 mix.
   - (ii) Pulse treatment for 6 hours (with and without S9 mix) followed by sampling at 24 hours.
   - (iii) Chromosome preparation for the presence of polyploid cells
   - (iv) Use of single sex (male) in rodent micronucleus test

ICH - International Conference on Harmonization

Pharmaceutical products. Where there is a cause for concern about the products existed, alternative relevant models have to be developed and these products should be tested. The outcome of genotoxicity tests are needed for the design and conduct of long term Carcinogenicity study and interpretation of the test results.

3.1. Principle of genotoxicity testing

Chemicals that exert their adverse effect through interaction with the genetic material (DNA) of cells are called genotoxic. Most human carcinogens are genotoxic in nature. The science of genotoxicity mainly concerns that chemicals, which induce
mutations in various experimental models, may conceivably affect the incidence of heritable mutations in man\textsuperscript{36,27}. Genotoxicity tests can be defined as \textit{in vitro} or \textit{in vivo} tests designed to detect drugs, which can induce genetic damage directly or indirectly by various mechanisms of action. Genotoxicity tests enable hazard identification with respect to DNA damage and its fixation in the form of gene mutations, large-scale chromosomal damage, recombination and numerical chromosome changes. Drugs that are positive in these tests that detect such kind of damage have the potential to be human carcinogens and/or mutagens \textit{i.e.}, may induce cancer and/or heritable defects.

3.2. The concept of test batteries

During the last two decades, several test systems with different end-points have been introduced successfully in routine genotoxicity testing\textsuperscript{36}. Three levels (gene, chromosome and genome) of information are required to give the comprehensive coverage of the mutagenic potential of a new drug substance\textsuperscript{36}. Genotoxicity tests currently available rarely detect more than one end-point in a single assay system\textsuperscript{36}. Hence, to minimize the risk factors, a NCE has to be subjected to a battery of genotoxicity tests. Genotoxic studies along with data on pharmacokinetic (PK) and toxicokinetic (TK) represent a more rational approach for the assessment of mutagens and carcinogens\textsuperscript{36}. Such information not only allows the correlation of toxic signs and symptoms with blood tissue levels of the agent, but also permits the selection of suitable animal species. A judicious test selection is essential to determine the risk potential of NCE completely.

Therefore, the usual approach is to perform a battery of \textit{in vitro} and \textit{in vivo} tests as part of the toxicological evaluation process. However, there is no universal agreement on the best combination of tests for a particular purpose. With the continued refinement on the global requirements for marketing new molecules by ICH, there has been a clear support and encouragement for obtaining multidisciplinary information in a given preclinical safety study for better interpretation of the data\textsuperscript{32}.

3.3. Criteria for test battery selection

For setting up of a battery of tests in genotoxicity testing, it has always been observed that \textit{in vitro} tests play a major role due to their high sensitivity and rapidity. Preliminary tests are designed in such a way that it can detect majority of the genotoxic carcinogens\textsuperscript{32}. One of the most important criteria considered for \textit{in vitro} test evaluation is the relative sensitivity of different cell lines and their genetic diversity\textsuperscript{34}. For assay reproducibility, a more comprehensive protocol with clear understanding of critical processes (pH shift, high osmolality, high ionic strength) involved during assay performance are necessary\textsuperscript{35}. The \textit{in vivo} test models are generally designed to see the chemical effects on the route of exposure, duration of treatment, metabolism and target organ exposure therapeutically relevant to humans.

3.4. Methods for genotoxicity screening

Different types of experimental protocol design and guidelines have been developed to conduct genotoxic evaluations\textsuperscript{36,37}. These methods include microbial and mammalian (both \textit{in vitro} and \textit{in vivo}) studies. It is appropriate to assess genetic toxicity initially in a bacterial test (Ames test). This test has been shown to detect point mutation. The original mutation was induced in the histidine operon of \textit{Salmonella typhimurium} and the bacteria cannot synthesize histidine of its own, unless there is a second reverse mutation induced by the chemical under investigation. Majority of genotoxic carcinogens can be detected by this test\textsuperscript{36,39}.

DNA damage considered relevant for mammalian cells cannot be adequately measured in bacteria and should be evaluated in mammalian cells. Several mammalian cell systems are in use and detect both gene mutation as well as gross chromosomal damage in \textit{in vitro} test system\textsuperscript{34}. The exogenous source of metabolic activation (S9 fraction) is added to culture condition to activate the indirectly acting chemicals. An \textit{in vivo} test provides the opportunity to evaluate a compound taking additional relevant factors (absorption, distribution, metabolism and excretion) into account, which may influence the genotoxic activity. So \textit{in vivo} tests permit the detection of some additional genotoxic agents. Usually an \textit{in vivo} test for chromosomal damage in rodent hemopoietic cells fulfils these requirements\textsuperscript{40-43}. This \textit{in vivo} test for chromosomal damage could be either an analysis of metaphase in bone marrow or an analysis of micronuclei in erythrocytes. Micronuclei represent fragments of chromosomes or entire chromosomes.
Table 2. Limitations in genetic toxicity tests in different models.

<table>
<thead>
<tr>
<th>Processes/Properties</th>
<th>Microbial</th>
<th>Mammalian (in vitro)</th>
<th>Mammalian (in vivo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2. Cytotoxicity</td>
<td>2. Extreme cytotoxicity can interfere in test result.</td>
<td>2. Extreme cytotoxicity can interfere in test result.</td>
<td>2. Uptake, metabolism and distribution of the test compound can alter the target organ toxicity.</td>
</tr>
<tr>
<td>3. Metabolism</td>
<td>3. Different sources of S9 and conditions of incubation can lead to qualitative and quantitative differences.</td>
<td>3. Different sources of S9 and conditions of incubation can lead to qualitative and quantitative differences.</td>
<td>3. Varies among strains/species/ sexes/tissues of the animal.</td>
</tr>
<tr>
<td>4. DNA repair/cell proliferation</td>
<td>4. Lack of DNA repair system exaggerated genotoxicity.</td>
<td>4. Selective cell proliferation by some chemicals increases DNA lesion by forming DNA adducts.</td>
<td>4. Some selective chemicals disturb DNA replication and delay in cell cycle; hence interfere in the end point evaluation.</td>
</tr>
<tr>
<td>5. Physiological differences</td>
<td>5. Prokaryotes cannot mimic the network and delicate intracellular biochemical pathways.</td>
<td>5. Limited enzymatic capabilities and viability.</td>
<td>5. In some cases, drug levels cannot reach the target organ because of distribution.</td>
</tr>
<tr>
<td>6. Cytogenetic end points</td>
<td>6. Only gene mutation can be detected in a single cell system.</td>
<td>6. Both gene as well as chromosomal changes in a single cell line.</td>
<td>6. Both gene as well as chromosomal evaluation in different tissues.</td>
</tr>
<tr>
<td>7. Dose/Conc. limitations</td>
<td>7. Test compounds solubility in culture medium is a limitation factor</td>
<td>7. Chemical solubility in culture medium is a limitation factor.</td>
<td>7. Varies among species/ sexes/tissues of the animal.</td>
</tr>
</tbody>
</table>

that do not get incorporated into the daughter nuclei during cell division. Drugs that have clastogenic (induce chromosomal breakage) as well as aneugenic activities (damage to the spindle apparatus) cause an increase in the micronucleus frequency. Numerical as well as structural chromosomal aberrations viz., breaks, gaps, deletion, exchanges and pulverization can be detected in bone marrow metaphase analysis. Positive and negative controls are concurrently used with the test compound. Low doses of positive compounds are generally tested to detect the sensitivity of test system. Historical positive and negative control data (provided these are homogenous) increase the statistical power of the test results. In most of the cases, the site and mechanism by which genotoxicity is produced by the compound under the study is 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 pertinent parameters or end points 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 (Table 2). 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.

3.5. Drawbacks of current genotoxicity tests

Majority of the currently used genotoxicity assays for regulatory toxicity testing were developed in 1970’s. Thus, their throughput cannot meet the requirements of the drug discovery requirements. In

4. Genesis and concept of ICH

The ICH harmonization process was first started in 1991 in Brussels, Germany. It is a tripartite agreement between European Community (EC), USA and Japan. It was started from the long drawn efforts of the International Association of Pharmaceutical
Technology (IAPT) to develop uniform guidelines and to involve all the major countries to agree on a single common approval for the new drugs. The main objective of ICH is to overcome the regional disparities and to recommend international standards for testing pharmaceuticals for pre-clinical safety studies. The other objective of the unification process is to bring different agencies to one platform with a common purpose and to provide high quality, safe and effective medicines to patients within a stipulated duration of time. In 1992, genotoxicity became an ICH topic and the genotoxicity working group identified more than 60 strategic and technical issues, which differed in substance between the regulatory authorities of the USA, the European Union and Japan. It was also noted that the importance given to various genotoxicity tests differed considerably from the guidelines of one country to another. It is important to know that whether any scientific justification exists for such importance given to various genotoxicity testing and would it be possible to develop a mutually acceptable guideline.

4.1 ICH guidelines

The fourth International Conference on Harmonization summarizes the pre-clinical testing guidelines for genotoxicity as well as other aspects of toxicity testing. It also fulfils the initial harmonization targets for the three ICH regions. From regulatory point of view an ordered approach using a limited number of well defined tests that complement each other in terms of end points and which permit a systematic assessment of genotoxicity is necessary. Recently, a unified approach has been made through the International Conference on Harmonization (ICH) consisting of representatives of pharmaceutical industry, scientists and regulatory authorities to develop uniform guidelines for toxicological testing. Out of the total 13 ICH safety guidelines, two were devoted to genotoxicity testing. The first genotoxicity-working group recommended the following two guidelines:


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.

I. In vitro Studies

i) The detection of mutagens, which induces DNA base changes, is enhanced by including strains that detects A-T base pair changes. This can be achieved by including either E. coli WP2 uvrA or the same strain containing the plasmid (pKM101) or Salmonella typhimurium TA102.

ii) Chemicals tested at highly toxic doses in vitro can lead to 'false positive' results. On the other hand, a genotoxin can be missed if not tested well up to the toxic range. Therefore, it was recommended that the test compound should be tested up to a concentration, which produces at least 50% inhibition in cell proliferation or mitotic index for cytogenetic tests.

iii) It has been observed that some genotoxins are only detectable when tested in to the insoluble range. To evaluate the genotoxicity of a compound without cytotoxicity, the maximum concentration of the test compound that can be used is 5 mg/plate and 10 mM for bacterial and mammalian cell culture respectively. In case of precipitation of the compound, the studies should be carried out up to the lowest concentration at which precipitation occurs.

The guideline also gives guidance on the interpretation of test results from in vitro tests. In particular, suggestion has been made on confounding factors that can lead to inaccurate interpretation of the data.

II. In vivo Studies

i) Both rats and mice are valid for in vivo detection of genotoxins.

ii) As per the test protocol recommendation bone marrow micronucleus test and metaphase analysis are interchangeable for regulatory purpose and only males are sufficient for detection of genotoxins.
### Table 3. ICH specific recommendations over the existing test protocols of US, EU and Japan regulatory authorities.

<table>
<thead>
<tr>
<th>The existing test protocol</th>
<th>The revised test protocol (as per ICH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Ames test detects changes at G-C base pairs. All genotoxic carcinogens may not be detected in the present tester strains.</td>
<td><strong>The S2 A guideline (in vitro tests)</strong>&lt;br&gt;1. Inclusion of a strain (<em>Salmonella typhimurium</em> TA 102), which detects at A-T base pair changes within DNA. Genotoxic carcinogen can be detected in this bacterial strain.</td>
</tr>
<tr>
<td>2. No specific recommendation has been made to detect DNA cross-linking agents.</td>
<td>2. Inclusion of <em>S. typhimurium</em> (TA 102) or to add a repair proficient <em>E. Coli</em> strain, such as WP2 pKM101.</td>
</tr>
<tr>
<td>3. No toxicity level defined in bacterial test.</td>
<td>3. In reverse mutation test, the highest concentration of test compound is desired to show evidence of significant toxicity.</td>
</tr>
<tr>
<td>4. High level of cytotoxicity <em>in vitro</em> which can interfere with the relevant genetic end point can increase the number of false positive.</td>
<td>4. The desired level of toxicity should be greater than 50% reduction in cell number or culture confluency. For lymphocyte culture, an inhibition of index by greater than 50% is considered sufficient.</td>
</tr>
<tr>
<td>5. For freely soluble, non-toxic compound the upper dose levels are not specified.</td>
<td>5. Freely soluble, non-toxic compounds, the desired upper treatment levels are 5 mg/plate for bacteria and 5 mg/ml or 10 mM (which is the lower) for mammalian cells.</td>
</tr>
<tr>
<td>6. For poorly soluble compounds no specific recommendation for top concentration are existing.</td>
<td>6. If no cytotoxicity is observed, then the lowest precipitating concentration should be used as the top concentration. If dose related cytotoxicity or mutagenicity is noted, irrespective of solubility, the top concentration should be based on toxicity as described above.</td>
</tr>
<tr>
<td>7. For <em>in vivo</em> test, US FDA prefers metaphase analysis (generation of chromosome damage) but in Europe and Japan, the preference has been for ‘micronucleus’ test.</td>
<td><strong>The S2B guideline (In vivo tests)</strong>&lt;br&gt;7. Either the analysis of chromosomal aberrations or the measurement of micronucleated polychromatic erythrocytes in peripheral blood is an acceptable alternative in the mouse.</td>
</tr>
<tr>
<td>8. Rodents of both sexes (male/female) are used in the routine bone marrow micronucleus test.</td>
<td>8. Male only sufficient. If gender specific drugs are to be tested, then normally animals of the corresponding sex should be used.</td>
</tr>
</tbody>
</table>

iii) In addition to the above two tests, micronucleus test in peripheral blood of mice is also acceptable for generating reproducible data.

When positive response is not obtained, it is necessary to validate the assay by demonstrating the exposure to the target tissue. This is particularly important when a 'positive result' has been obtained in *in vitro* genotoxicity tests.

(b) S2B guideline

The S2B guideline covers the identification of a standard set of tests to be conducted for registration and the extent of confirmatory experimentation in *in vitro* genotoxicity tests in the standard test battery.

The ICH has recommended three standard test batteries to evaluate all the pharmaceutical products. The modifications of test protocols by ICH over the existing protocols are summarized in Table 3. Based upon the ICH considerations, the following three standard test batteries are recommended:

**A test for gene mutation in bacteria.**

An *in vitro* test with evaluation of chromosomal damage in mammalian cells or an *in vitro* mouse lymphoma tk assay.

An *in vivo* test for chromosomal damage using rodent haemopoietic cells.

It is most appropriate to test genetic toxicity in a bacterial reverse mutation (Ames test) assay to get
preliminary information. This test has been shown to detect base substitution, frame shift point mutations as well as DNA cross-linking agents. Majority of genotoxic carcinogens can be detected by this test due to the relative sensitivity of the tester strains to different test compounds and the observation of mutation, which occurs at a very low concentration. The in vitro tests employing different mammalian cells play a major role, since they are highly sensitive and majority of genotoxic compounds can be detected. For poor bioavailable compounds, it is difficult to achieve target organ exposure in mammalian bone marrow cells. This can be overcome by directly exposing the cells to chemicals in in vitro system. However, an in vivo test battery is usually considered as a part of the basic test battery to provide additional relevant factors (absorption, distribution, metabolism and excretion) that may influence the genotoxic activity of a compound. So by testing a compound in the three test batteries recommended by ICH, both gene as well as chromosomal level information can be obtained. Again, there will be least chance of getting false positive and negative result for a NCE under investigation.

4.2. Modification of the test battery

There are certain situations where the standard 3-test battery may need further modification.

i) Compounds, which are highly toxic

Compounds, which are highly toxic to bacteria and interfere with mammalian test system(s) should be tested in two in vitro mammalian cell tests using two different cell types. The end point should include determination of gene mutation and chromosome damage.

ii) Compounds bearing structural alerts

The compounds, which produce negative results in the standard genotoxic test battery but having structural alerts, need to be subjected to further additional tests with modified protocols.

iii) Compounds, which are not absorbed

Compounds, which are not systemically absorbed and therefore not available to the target tissue (bone marrow or liver), should be solely tested in in vitro assays. These tests should include a bacterial gene mutation and two in vitro mammalian cell assays using two different cell types with two different end points.

iv) Evidences of tumour response

The compounds, which are negative in standard genotoxic test battery but exhibit carcinogenic potential, should be further tested in appropriate models to evaluate the mechanism of action associated with the carcinogenic activity. Additional testing can include exogenous metabolic activation or can include (a) the liver unscheduled DNA synthesis (UDS) test, (b) 32P post labelling (c) mutation induction in transgenes and (d) molecular characterisation of genetic changes in tumour related genes.

v) Structurally unique chemical classes

On rare occasions, a completely novel compound in a unique chemical class will be introduced as a pharmaceutical. When such compounds, which are not subjected to chronic rodent carcinogenicity bioassays, may be tested further, by employing genotoxicity testing using additional in vitro and/or in vivo assays.

4.3. Specific recommendation

I. In vitro test results

The ICH Expert Working Group’s recommendations are mainly based upon the latest OECD guidelines. The in vitro gene mutation tests in both bacterial and mammalian system should include range finding results which will guide the selection of appropriate concentration to be used in the definitive mutagenicity test. The range finding tests in bacteria are performed on all strains with and without metabolic activation with appropriate positive and negative controls. In ICH recommendation, the mouse lymphoma tk assay (MLA) is regarded superior with several reasons than test using hgprt locus test in mice. In mouse lymphoma tk assay for short exposure (3 to 4hours), the test should include both with and without metabolic activation with appropriate positive and negative controls. In case of negative results without metabolic activation, a continuous exposure for approximately 24 hours is needed. A negative in vitro result may be further tested with specific external
meta-bolizing system, which is known to be competent for the metabolism/activation of that specific class of compound under test. The in vitro chromosomal damage should be performed as per the protocol mentioned in in vitro gene mutation test with additional information of polyploidy and mitotic index induced by the chemical under investigation.

There are several situations, which can lead to a positive in vitro test results and these should be further evaluated for their biological relevance. The increase in response over negative control should indicate a meaningful genotoxic effect to the target cells. Further investigation should be made to demonstrate that the in vitro positive results are not originated due to the extreme conditions (e.g., excess of pH, osmolality, heavy precipitate in suspension culture). Clear evidence has to be established that the in vitro positive results did not originate due to contaminant or a metabolite from in vitro specific metabolic pathways. The ICH expert working group has described a series of conditions from which positive results of questionable biological relevance might be obtained in mammalian cells in vitro.

II. In vivo test results

The chromosomal aberration assay in rodent bone marrow nucleated cells can detect a wide spectrum of changes, which result from breakage of one or more chromatids as the initial event. Breakage of chromatids or chromosomes can result into micronucleus formation if an acentric fragment is produced. Therefore, assays detecting either chromosomal aberration or micronuclei are acceptable for detecting clastogens. Therefore, the measurement of micronucleated erythrocytes in polychromatic erythrocytes is an acceptable alternative to detect clastogens/aneuploidy inducers. Male mice are more sensitive than female mice for induction of micronuclei and the differences are only quantitative, but not qualitative.

III. Detection of germ cell mutagens

With respect to the detection of germ cell mutagens, no specific test has been recommended by ICH guidelines. It is well recognised that mutations in the germ line cells mainly lead to improper function of germ cells and early embryo loss. Among the abnormalities, aneuploidy is the most common followed by polyploidy. Structural abnormalities constitute about 5 percent of the total germ cell disorders. There may be specific types of mutagens, e.g., aneuploidy inducers, which act preferentially during meiotic gametogenesis stages. When assessing the likelihood of a genotoxic potential of the germ cell mutagen, the type of active compound, pharmacokinetic data and indication for medical use are taken into account. This could lead to a consideration that a genetic hazard for human germ cells is not to be expected.

4.4. Additional requirements for specific situation

Though male mice bone marrow micronucleus test is sufficient for in vivo genotoxicity testing, in some specific cases the female mice as well as the rats can be used to ensure the authenticity of genotoxicity testing. Reports indicate that the metabolism of the compounds may vary not only within the sexes of same species but also across the species. No detailed discussion has been made under ICH guidelines for in vivo gene mutation assay using the transgenic mice model. Some specific mutagens e.g., aneuploidy inducers, act preferentially during meiotic gametogenesis stages and there is no conclusive experimental method exists till date to detect these substances. There are many chemicals producing positive results in in vitro but not in in vivo test system hence are not relevant in terms of human exposure. So it will be a greater need for mechanistic studies in order to see whether the aberration induced in vitro are due to a process with or without a threshold.

4.5. Impact of ICH guidelines

The harmonization process will increase the speed of existing regulatory process for NCE. It also eliminates the drawbacks in the current toxicological evaluation of new drugs. This will ultimately improve the accuracy of genotoxicity detection and help to conserve resources. With the introduction of ICH, it has been suggested that all genotoxicity tests should be carried out early in the development of pharmaceuticals and should be completed prior to the initiation of phase II clinical trials. The guidelines aim to be flexible for new group of pharmaceuticals, especially biotechnology derived products and allow the
substitution of newer generation of validated bioassays. Once the ICH guidelines are implemented, the process of approval by the regulatory authorities will be streamlined and reduce the time and conserve the resources in drug dis-covery and development programme.

5. New methods in genotoxicity testing

In general, the three standard genotoxicity test battery is adequate for evaluation of genotoxicity of a 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 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 $^{32}$P-post labelling, 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 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.

6. Conclusion

Now-a-days drug discovery and development is rapid, time saving and productive due to the use of newer technologies such as genomics, high throughput screening and proteomics. Pharmaceutical companies and regulatory agencies are preparing themselves to meet the challenges of the 21st century and toxicologists and other scientists need to re-evaluate the existing protocols in the changing environment. The existing guidelines, in a developing country like India need to be re-evaluated and modified in accordance to the newer trends of globalization. By adopting the ICH guidelines, the process of new drug approval can be streamlined.

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HIGH FIBRE DIET LINKED TO GOOD MENTAL HEALTH

Having a high fibre diet has been linked to a ‘happier’ state of mind, increased energy levels and faster thought processes, according to the results of a recent study published in the Journal Appetite. Researchers based at Cardiff University conducted a study to examine the effect of breakfast cereals on human fatigue. Volunteers aged between 30-80 years were recruited for a 4-week period in which they had to ingest 40g of high fibre cereal each day. After just one week, researchers noted stark differences in terms of a 10% reduction in fatigue, lower depression scores and better cognitive powers. The Authors conclude that although the physical benefits of a high-fibre diet have been widely acknowledged amongst healthcare professionals for many years, this is the first time high-fibre intake has been associated with improved mental health. And stressed the need for further research into their findings.
There is no requirement in the revised guideline for a second assay when the result is clearly either negative or positive. Experience with testing pharmaceuticals has shown that there is rarely any advantage in the pre-incubation method[5]. For the single test, therefore, either the plate-incorporation or pre-incubation options will be equally accepted. The new guideline provides two options for a genotoxicity test battery. The test battery approach is designed to reduce the risk of false negative results for substances with genotoxic potential as well as reducing false positives. References: 1. ICH Guideline S2A. Guidance on Specific Aspects of Regulatory Genotoxicity Tests for Pharmaceuticals. 1995. 2. ICH Guideline S2B. Regulatory authorities require data from acute toxicity studies for the registration of any pharmaceutical intended for human use. Traditionally, the information obtained from these studies has been used to set an appropriate dose level for repeat dose studies in animals and to support the effects of overdose in humans. There was general agreement that if tests were only being done for a regulatory purpose with no sound scientific justification then they should not be conducted. 5. Sally Robinson et al in preparation. 6. ICH M3: Guidance on non-clinical safety studies for the conduct of human clinical trials for pharmaceuticals (1997). May 2007 Challenging requirement for acute toxicity studies: A workshop report. 9. Toxicity testing S4 Single dose toxicity tests Agreement was reached, at the time of ICH 1, in 1991, that the determination of the median lethal dose (LD50) should be abandoned for pharmaceuticals. The recommendation was published in the Proceedings of the First International Conference on Harmonisation, p. 184. S4A Duration of chronic toxicity testing in animals (rodent and non-rodent). Clinical safety E1 The extent of population exposure to assess clinical safety for drugs intended for long-term treatment of non-life-threatening conditions E2A Clinical safety data management: definitions and standards for expedited reporting E2B/ Maintenance of the clinical safety data management including the M2 maintenance of the electronic transmission of individual case safety reports message.