INTERNATIONAL CONFERENCE ON HARMONISATION OF TECHNICAL REQUIREMENTS FOR REGISTRATION OF PHARMACEUTICALS FOR HUMAN USE

ICH HARMONISED TRIPARTITE GUIDELINE

GUIDANCE ON SPECIFIC ASPECTS OF REGULATORY GENOTOXICITY TESTS FOR PHARMACEUTICALS

Recommended for Adoption

at Step 4 of the ICH Process

on 19 July 1995

by the ICH Steering Committee

This Guideline has been developed by the appropriate ICH Expert Working Group and has been subject to consultation by the regulatory parties, in accordance with the ICH Process. At Step 4 of the Process the final draft is recommended for adoption to the regulatory bodies of the European Union, Japan and USA.

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1. INTRODUCTION

Guidelines for the testing of pharmaceuticals for genetic toxicity have been established in the European Community (EEC, 1987) and Japan (Japanese Ministry of Health and Welfare, 1989). FDA´s Centers for Drugs and Biologics Evaluation and Research (CDER and CBER) currently consider the guidance on genetic toxicity testing provided by the FDA Center for Food Safety and Applied Nutrition (Federal Register notice, March 29, 1993) to be applicable to pharmaceuticals.

The following notes for guidance should be applied in conjunction with existing guidelines in the USA, the European Community, and Japan. The recommendations below are derived from considerations of historical information held within the international pharmaceutical industry; the three regulatory bodies and the scientific literature. Where relevant the recommendations from the latest review of OECD guidelines (OECD, 1994) and the 1993 International Workshop on Standardisation of Genotoxicity Test Procedures (Mutation Research No. 312(3), 1994) have been considered.

2. SPECIFIC GUIDANCE AND RECOMMENDATIONS

2.1 Specific guidance for *in vitro* tests

2.1.1 The base set of strains used in bacterial mutation assays

Current guidelines for the detection of bacterial mutagens employ several strains to detect base substitution and frameshift point mutations. The *Salmonella typhimurium* strains mentioned in guidelines (normally TA1535, TA1537, TA98 and TA100) will detect such changes at G-C (guanine-cytosine) sites within target histidine genes. It is clear from the literature that some mutagenic carcinogens also modify A-T (adenine-thymine) base pairs. Therefore the standard set of strains used in bacterial mutation assays should include strains that will detect point mutations at A-T sites, such as *Salmonella typhimurium* TA102, which detects such mutations within multiple copies of *hisG* genes or *Escherichia coli* WP2 *uvrA*, which detects these mutations in the *trpE* gene or the same strain possessing the plasmid (pKM101), which carries *mucAB* genes that enhance error prone repair (see note 1). In conclusion, the following base set of bacterial strains should be used for routine testing: the strains cited below are all *Salmonella typhimurium* isolates, unless specified otherwise.

1. TA98; 2. TA100; 3. TA1535; 4. TA1537 or TA97 or TA97a (see note 2); 5. TA102 or *Escherichia coli* WP2 *uvrA* or *Escherichia coli* WP2 *uvrA* (pKM101).

In order to detect cross-linking agents it may be preferable to select *Salmonella typhimurium* TA 102 or to add a repair proficient *Escherichia coli* strain, such as WP2 pKM101. It is noted that such compounds are detected in assays that measure chromosome damage.

2.1.2. Definition of the top concentration for *in vitro* tests

2.1.2.1. High concentration for non-toxic compounds

For freely soluble, non-toxic compounds, the desired upper treatment levels are 5 mg/plate for bacteria and 5 mg/ml or 10 mM (whichever is the lower) for mammalian cells.

2.1.2.2. Desired level of cytotoxicity

Some genotoxic carcinogens are not detectable in *in vitro* genotoxicity assays unless the concentrations tested induce some degree of cytotoxicity. It is also apparent that excessive toxicity often does not allow a proper evaluation of the relevant genetic endpoint. Indeed at very low survival levels in mammalian cells, mechanisms other than direct genotoxicity *per se* can lead to 'positive' results that are related to cytotoxicity and not genotoxicity (e.g., events associated with apoptosis, endonuclease release from lysosomes etc.). Such events are likely to occur once a certain concentration threshold is reached for a toxic compound.

To balance these conflicting considerations the following levels of cytotoxicity are currently acceptable for *in vitro* bacterial and mammalian cell tests (concentrations should not exceed the levels specified in 2.1.2.1.):

i) In the bacterial reverse mutation test, the highest concentration of test compound is desired to show evidence of significant toxicity. Toxicity may be detected by a reduction in the number of revertants, a clearing or diminution of the background lawn.

ii) The desired level of toxicity for *in vitro* cytogenetic tests using cell lines should be greater than 50% reduction in cell number or culture confluency. For lymphocyte cultures, an inhibition of mitotic index by greater than 50% is considered sufficient.

iii) In mammalian cell mutation tests ideally the highest concentration should produce at least 80% toxicity (no more than 20% survival). Toxicity can be measured either by assessment of cloning efficiency (e.g., immediately after treatment), or by calculation of relative total growth, i.e., the product of relative suspension growth during the expression period and relative plating efficiency at the time of mutant selection. Caution is due with positive results obtained at levels of survival lower than 10%.

2.1.2.3. Testing of poorly soluble compounds

There is some evidence that dose-related genotoxic activity can be detected when testing certain compounds in the insoluble range in both bacterial and mammalian cell genotoxicity tests. This is generally associated with dose-related toxicity (see note 3). It is possible that solubilisation of a precipitate is enhanced by serum in the culture medium or in the presence of S9-mix constituents. It is also probable that cell membrane lipid can facilitate absorption of lipophilic compounds into cells. In addition some types of mammalian cells have endocytic activity (e.g., Chinese hamster V79; CHO and CHL cells) and can ingest solid particles which may subsequently disperse into the cytoplasm. An insoluble compound may also contain soluble genotoxic impurities. It should also be noted that a number of insoluble pharmaceuticals are administered to humans as suspensions or as particulate materials.

On the other hand heavy precipitates can interfere with scoring the desired parameter and render control of exposure very difficult (e.g., where a centrifugation step(s) is included in a protocol to remove cells from exposure media) (see note 4); or render the test compound unavailable to enter cells and interact with DNA.

The following strategy is recommended for testing relatively insoluble compounds. The recommendation below refers to the test article in the culture medium.

If no cytotoxicity is observed then the lowest precipitating concentration should be used as the top concentration but not exceeding 5 mg/plate for bacterial tests and 5 mg/ml or 10 mM for mammalian cell tests. If dose-related cytotoxicity or mutagenicity is noted, irrespective of solubility, then the top concentration should be based on toxicity as described above. This may require the testing of more than one precipitating concentration (not to exceed the above stated levels). It is recognised that the desired levels of cytotoxicity may not be achievable if the extent of precipitation interferes with the scoring of the test. In all cases precipitation should be evaluated at the beginning and at the end of the treatment period using the naked eye.

2.2. Specific guidance for *in vivo* tests

2.2.1. Acceptable bone marrow tests for the detection of clastogens *in vivo*

Tests measuring chromosomal aberrations in nucleated bone marrow cells in rodents can detect a wide spectrum of changes in chromosomal integrity. These changes almost all result from breakage of one or more chromatids as the initial event. Breakage of chromatids or chromosomes can result in micronucleus formation if an acentric fragment is produced; therefore assays detecting either chromosomal aberrations or micronuclei are acceptable for detecting clastogens (see note 5). Micronuclei can also result from lagging of one or more whole chromosome(s) at anaphase and thus micronucleus tests have the potential to detect some aneuploidy inducers (see note 6).

In conclusion either the analysis of chromosomal aberrations in bone marrow cells or the measurement of micronucleated polychromatic erythrocytes in bone marrow cells *in vivo* is acceptable for the detection of clastogens. The measurement of micronucleated immature (e.g., polychromatic) erythrocytes in peripheral blood is an acceptable alternative in the mouse, or in any other species in which the inability of the spleen to remove micronucleated erythrocytes has been demonstrated, or which has shown an adequate sensitivity to detect clastogens/aneuploidy inducers in peripheral blood (see note 7).

2.2.2. Use of male/female rodents in bone marrow micronucleus tests

Extensive studies of the activity of known clastogens in the mouse bone marrow micronucleus test have shown that in general male mice are more sensitive than female mice for micronucleus induction (see note 8). Quantitative differences in micronucleus induction have been identified between the sexes, but no qualitative differences have been described. Where marked quantitative differences exist, there is invariably a difference in toxicity between the sexes. If there is a clear qualitative difference in metabolites between male and female rodents, then both sexes should be used. Similar principles can be applied for other established *in vivo* tests (see note 9). Both rats and mice are deemed acceptable for use in the bone marrow micronucleus test (see note 10).

In summary, unless there are obvious differences in toxicity or metabolism between male and female rodents, then males alone are sufficient for use in bone marrow micronucleus tests. If gender-specific drugs are to be tested, then normally animals of the corresponding sex should be used.

2.3. Guidance on the evaluation of test results

Comparative trials have shown conclusively that each *in vitro* test system generates both false negative and false positive results in relation to predicting rodent carcinogenicity. Genotoxicity test batteries (of *in vitro* and *in vivo* tests) detect carcinogens that are thought to act primarily via a mechanism involving direct genetic damage, such as the majority of known human carcinogens. Therefore, these batteries may not detect non-genotoxic carcinogens. Experimental conditions, such as the limited capability of the *in vitro* metabolic activation systems, can also lead to false negative results in *in vitro* tests. The test battery approach is designed to reduce the risk of false negative results for compounds with genotoxic potential, while a positive result in any assay for genotoxicity does not necessarily mean that the test compound poses a genotoxic/carcinogenic hazard to humans.

2.3.1. Guidance on the evaluation of *in vitro* test results

2.3.1.1. *In vitro* positive results

The scientific literature gives a number of conditions which may lead to a positive *in vitro* result of questionable relevance. Therefore, any *in vitro* positive test result should be evaluated for its biological relevance taking into account the following considerations (this list is not exhaustive, but is given as an aid to decision-making):

i) Is the increase in response over the negative or solvent control background regarded as a meaningful genotoxic effect for the cells?

ii) Is the response concentration-related?

iii) For weak/equivocal responses, is the effect reproducible?

iv) Is the positive result a consequence of an *in vitro* specific metabolic activation pathway/*in vitro* specific active metabolite (see also note 12)?

v) Can the effect be attributed to extreme culture conditions that do not occur in *in vivo* situations, e.g., extremes of pH; osmolality; heavy precipitates especially in cell suspensions (see note 4)?

vi) For mammalian cells, is the effect only seen at extremely low survival levels (see section 2.1.2.2. for acceptable levels of toxicity)?

vii) Is the positive result attributable to a contaminant (this may be the case if the compound shows no structural alerts or is weakly mutagenic or mutagenic only at very high concentrations)?

viii) Do the results obtained for a given genotoxic endpoint conform to that for other compounds of the same chemical class?

2.3.1.2. *In vitro* negative results

For *in vitro* negative results special attention should be paid to the following considerations (the examples given are not exhaustive, but are given as an aid to decision-making): Does the structure or known metabolism of the compound indicate that standard techniques for *in vitro* metabolic activation (e.g., rodent liver S9) may be inadequate? Does the structure or known reactivity of the compound indicate that the use of other test methods/systems may be appropriate?

2.3.2. Guidance on the evaluation of *in vivo* test results

In vivo tests, by their nature, have the advantage of taking into account absorption, distribution and excretion, which are not factors in *in vitro* tests, but are relevant to human use. In addition metabolism is likely to be more relevant *in vivo* compared to the systems normally used *in vitro*. There are a few validated *in vivo* models accepted for assessment of genotoxicity. These include the bone marrow or peripheral blood cytogenetic assays. If a compound has been tested *in vitro* with negative results, it is usually sufficient to carry out a single *in vivo* cytogenetics assay.

For a compound that induces a biologically relevant positive result in one or more *in vitro* tests (see section 2.3.1.1.), a further *in vivo* test in addition to the *in vivo* cytogenetic assay, using a tissue other than the bone marrow/peripheral blood, can provide further useful information. The target cells exposed *in vivo* and possibly the genetic endpoint measured *in vitro* guide the choice of this additional *in vivo* test. However, there is no validated, widely used *in vivo* system which measures gene mutation. *In vivo* gene mutation assays using endogenous genes or transgenes in several tissues of the rat and mouse are at various stages of development. Until such tests for mutation become accepted, results from other *in vivo* tests for genotoxicity in tissues other than the bone marrow can provide valuable additional data but the assay of choice should be scientifically justified (see note 11).

If *in vivo* and *in vitro* test results do not agree, then the differences should be considered/explained on a case-by-case basis (see sections 2.3.1.1. and 2.3.2.1., and note 12).

In conclusion, the assessment of the genotoxic potential of a compound should take into account the totality of the findings and acknowledge the intrinsic values and limitations of both *in vitro* and *in vivo* tests.

2.3.2.1. Principles for demonstration of target tissue exposure for negative *in vivo* test results

In vivo tests have an important role in genotoxicity test strategies. The significance of *in vivo* results in genotoxicity test strategies is directly related to the demonstration of adequate exposure of the target tissue to the test compound. This is especially true for negative *in vivo* test results and when *in vitro* test(s) have shown convincing evidence of genotoxicity. Although a dose sufficient to elicit a biological response (e.g., toxicity) in the tissue in question is preferable, such a dose could prove to be unattainable since dose-limiting toxicity can occur in a tissue other than the target tissue of interest. In such cases, toxicokinetic data can be used to provide evidence of bioavailability. If adequate exposure cannot be achieved e.g., with compounds showing very poor target tissue availability, extensive protein binding etc., conventional *in vivo* genotoxicity tests may have little value.

The following recommendations apply to bone marrow cytogenetic assays, as examples; if other target tissues are used, similar principles should be applied.

For compounds showing positive results in any of the *in vitro* tests employed demonstration of *in vivo* exposure should be made by any of the following measurements:

i) By obtaining a significant change in the proportion of immature erythrocytes among total erythrocytes in the bone marrow, at the doses and sampling times used in the micronucleus test or by measuring a significant reduction in mitotic index for the chromosomal aberration assay.

ii) Evidence of bioavailability of drug related material either by measuring blood or plasma levels (see note 13).

iii) By direct measurement of drug-related material in bone marrow.

iv) By autoradiographic assessment of tissue exposure.

For methods ii) to iv), assessments should be made preferentially at the top dose or other relevant doses using the same species/strain and dosing route used in the bone marrow assay.

If *in vitro* tests do not show genotoxic potential, *in vivo* (systemic) exposure should be demonstrated and can be achieved by any of the methods above, but can also be inferred from the results of standard absorption, distribution, metabolism and excretion (ADME) studies in rodents.

2.3.2.2. Detection of germ cell mutagens

With respect to the detection of germ cell mutagens, results of comparative studies have shown that, in a qualitative sense, most germ cell mutagens are likely to be detected as such in somatic cell tests and negative results of *in vivo* somatic cell genotoxicity tests generally indicate the absence of germ cell effects (see note 14).

3. NOTES

1) Relevant examples of genotoxic carcinogens that are detected if bacterial strains with A-T target mutations are included in the base set can be found in the literature (e.g., Levin et al., 1983; Wilcox et al., 1990). Analysis of the database held by the Japanese Ministry of Labour on 5526 compounds (and supported by smaller databases held by various pharmaceutical companies), has shown that approximately 7.5% of the bacterial mutagens identified are detected by *E. coli* WP2 *uvrA*, but not by the standard set of four Salmonella strains. Although animal carcinogenicity data are not available on these compounds, it is likely that such compounds would carry the same carcinogenic potential as mutagens inducing changes in the standard set of Salmonella strains.

2) TA1537, TA97 and TA97a all contain cytosine runs at the mutation sensitive site within the relevant target histidine loci and show similar sensitivity to frameshift mutagens that induce deletion of bases in these frameshift hotspots. There was consensus agreement at the International Workshop on Standardisation of Genotoxicity Procedures, Melbourne, 1993, (Gatehouse et al., 1994) that all three strains could be used interchangeably.

3) Laboratories in Japan carrying out genotoxicity tests have much experience in testing precipitates and have identified examples of substances that are clearly genotoxic only in the precipitating range of concentrations. These compounds include polymers and mixtures of compounds; some polycyclic hydrocarbons; some phenylene diamines; heptachlor etc. Collaborative studies with some of these compounds have shown that they may be detectable in the soluble range, however it does seem clear that genotoxic activity increases well into the insoluble range. A discussion of these factors is given in the report of the '*in vitro*' sub group of the International Workshop on Standardisation of Genotoxicity Procedures, Melbourne, 1993 (Kirkland, 1994).

4) Testing compounds in the precipitating range is problematical with respect to defining the exposure periods for assays where the cells grow in suspension. After the defined exposure period, the cells are normally pelleted by centrifugation and are then resuspended in fresh medium without the test compound. If a precipitate is present, the compound will be carried through to the later stages of the assay making control of exposure impossible. If such cells are used e.g., human peripheral lymphocytes or mouse lymphoma cells, it is reasonable to use the lowest precipitating concentration as the highest tested.

5) As the mechanisms of micronucleus formation are related to those inducing chromosomal aberrations (e.g., Hayashi et al., 1984 and 1994; Hayashi, 1994), both micronuclei and chromosomal aberrations can be accepted as assay systems to screen for clastogenicity induced by test compounds. Comparisons of data where both the mouse micronucleus test and rat bone marrow metaphase analysis have been carried out on the same compounds have shown impressive correlation both qualitatively i.e., detecting clastogenicity and quantitatively i.e., determination of the lowest clastogenic dose. Even closer correlations can be expected where the data are generated in the same species.

6) Although micronuclei can arise from lagging whole chromosomes following interaction of a compound with the spindle apparatus, the micronucleus test may not detect all aneuploidy inducers. Specific aneuploidy assays may become available in the near future. One approach is the evolving rapid and sensitive technique for identifying individual (rodent) chromosomes in interphase nuclei, e.g., via fluorescence in situ hybridisation (FISH).

7) The peripheral blood micronucleus test in the mouse using acridine orange supravital staining was originally introduced by Hayashi et al. (1990). The test has been the subject of a major collaborative study by the Japanese Collaborative Study Group for the Micronucleus Test (Mutation Research, 278, 1992, Nos. 2/3). The tests were carried out in CD-1 mice using 23

test substances of various modes of action. Peripheral blood sampled from the same animal was examined 0, 24, 48 and 72 hours (or longer) after treatment. As a rule one chemical was studied by two different laboratories (46 laboratories took part). All chemicals were detected as inducers of micronuclei. There were quantitative differences between laboratories, but no qualitative differences. Most chemicals gave the greatest response 48 hours after treatment. Thus the results suggest that the peripheral blood micronucleus assay using acridine orange supravital staining can generate reproducible and reliable data to evaluate the clastogenicity of chemicals. Based on these data, the International Workshop on Standardisation of Genotoxicity Procedures, Melbourne, 1993 concluded that this assay is equivalent in accuracy to the bone marrow micronucleus assay (Hayashi et al., 1994). The application of the peripheral blood micronucleus assay to rats is under validation by the Japanese Collaborative Study Group for the Micronucleus Test.

8) A detailed collaborative study was carried out indicating that in general male mice were more sensitive than female mice for micronucleus induction, but where differences were seen they were only quantitative and not qualitative (The Collaborative Study Group for the Micronucleus Test, 1986). This analysis has been extended by the group considering the micronucleus test at the International Workshop on Standardisation of Genotoxicity Procedures, Melbourne, 1993 and having analysed data on 53 *in vivo* clastogens (and 48 non-clastogens), the same conclusions were drawn (Hayashi et al., 1994).

9) As the induction of micronuclei and chromosomal aberrations are related, it is reasonable to assume that the same conditions can be applied when using male animals in bone marrow chromosomal aberration assays. The peripheral blood micronucleus test has been validated only in male rodents (The Collaborative Study Group for the Micronucleus Test, 1992) as has the *ex vivo* UDS test (Kennely et al., 1993; Madle at al., 1994).

10) Both the rat and mouse are suitable species for use in the micronucleus test with bone marrow. However data are accumulating to show that some species specific carcinogens are species specific genotoxins (e.g., Albanese et al., 1988). When more data have accumulated there may be a case for carrying out micronucleus tests in both the rat and the mouse.

11) Apart from the cytogenetic assays in bone marrow cells, a large database for *in vivo* assays exists for the liver unscheduled DNA synthesis (UDS) assay (Madle et al., 1994). A review of the literature shows that a combination of the liver UDS test and the bone marrow micronucleus test will detect most genotoxic carcinogens with few false positive results (Tweats, 1994). False negative results with this combination of assays have been generated for some unstable genotoxic compounds and certain aromatic amines which are problematical for most existing *in vivo* screens (Tweats, 1994). Therefore, further *in vivo* testing should not be restricted to liver UDS tests as other assays may be more appropriate (e.g., ^{32}P post-labelling; DNA strand-breakage assays etc.), depending on the compound in question. It is important to recognise that for these *in vivo* endpoints, their relationship to mutation is not precisely known.

12) Examples to consider regarding the difference between *in vitro* and *in vivo* test results have been described in the literature. They include: (i) an active metabolite produced *in vitro* may not be produced *in vivo*, (ii) an active metabolite may be rapidly detoxified *in vivo* but not *in vitro*, (iii) rapid and efficient excretion of a compound may occur *in vivo* etc. Examples such as these have been described (e.g., Ashby, 1983).

13) The bone marrow is a well perfused tissue and it can be deduced therefore that levels of drug related materials in blood or plasma will be similar to those observed in bone marrow. This is borne out by direct comparisons of drug levels in the two compartments for a large series of different pharmaceuticals (Probst, 1994). Although drug levels are not always the same, there is

sufficient correlation for measurements in blood or plasma to be adequate for validating bone marrow exposure.

14) There may be specific types of mutagens, e.g., aneuploidy inducers, which act preferentially during meiotic gametogenesis stages. There is no conclusive experimental evidence for the existence of such substances to date.

4. GLOSSARY

Aneuploidy: numerical deviation of the modal number of chromosomes in a cell or organism.

Base substitution: the substitution of one or more base(s) for another in the nucleotide sequence. This may lead to an altered protein.

Cell proliferation: the ability of cells to divide and to form daughter cells.

Clastogen: an agent that produces structural changes of chromosomes, usually detectable by light microscopy.

Cloning efficiency: the efficiency of single cells to form clones. Usually measured after seeding low numbers of cells in a suitable environment.

Culture confluency: a quantification of the cell density in a culture (cell proliferation is usually inhibited at high degrees of confluency).

Frameshift mutation: a mutation (change in the genetic code) in which one base or two adjacent bases are added (inserted) or deleted to the nucleotide sequence of a gene. This may lead to an altered or truncated protein.

Gene mutation: a detectable permanent change within a single gene or its regulating sequences. The changes may be point mutations, insertions, deletions.

Genetic endpoint: the precise type or type class of genetic change investigated (e.g., gene mutations, chromosomal aberrations, DNA-repair, DNA-adduct formation, etc.).

Genetic toxicity, genotoxicity: a broad term that refers to any deleterious change in the genetic material regardless of the mechanism by which the change is induced.

Micronucleus: particle in a cell that contains microscopically detectable nuclear DNA; it might contain a whole chromosome(s) or a broken centric or acentric part(s) of chromosome(s). The size of a micronucleus is usually defined as being less than 1/5 but more than 1/20 of the main nucleus.

Mitotic index: percentage of cells in the different stages of mitosis amongst the cells not in mitosis (interphase) in a preparation (slide).

Plasmid: genetic element additional to the normal bacterial genome. A plasmid might be inserted into the host chromosome or form an extrachromosomal element.

Point mutations: changes in the genetic code, usually confined to a single DNA base pair.

Polychromatic erythrocyte: an immature erythrocyte in an intermediate stage of development that still contains ribosomes and, as such, can be distinguished from mature normochromatic erythrocytes (lacking ribosomes) by stains selective for ribosomes.

Survival (in the context of mutagenicity testing): proportion of cells in a living stage among dead cells, usually determined by staining and colony counting methods after a certain treatment interval.

Unscheduled DNA synthesis (UDS): DNA synthesis that occurs at some stage in the cell cycle other than S-phase in response to DNA damage. It is usually associated with DNA excision repair.

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