Guidance for Industry

S2(R1) Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Drug Evaluation and Research (CDER)
Center for Biologics Evaluation and Research (CBER)

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ICH

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Contains Nonbinding Recommendations

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I. INTRODUCTION (1)[2]

A. Objectives of the Guidance (1.1)

This guidance combines and replaces two ICH guidances, *S2A Specific Aspects for Regulatory Genotoxicity Tests for Pharmaceuticals* (ICH S2A guidance) and *S2B Genotoxicity: A Standard Battery for Genotoxicity Testing of Pharmaceuticals* (ICH S2B guidance). The purpose of the revision is to provide guidance on optimizing the standard genetic toxicology battery for prediction of potential human risks, and on interpreting results, with the goal of improving risk characterization for carcinogenic effects that have their basis in changes in the genetic material. The revised guidance describes internationally agreed-upon standards for follow-up testing and interpretation of positive results in vitro and in vivo in the standard genetic toxicology battery, including assessment of nonrelevant findings. This guidance is intended to apply only to products being developed as human pharmaceuticals.

B. Background (1.2)

The recommendations from the latest Organization for Economic Co-operation and Development (OECD) guidelines and the reports from the International Workshops on Genotoxicity Testing (IWGT) have been considered where relevant. In certain cases, the recommendations in this

[1] This guidance was developed within the Safety Implementation Working Group of the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and has been subject to consultation by the regulatory parties, in accordance with the ICH process. This document has been endorsed by the ICH Steering Committee at *Step 4* of the ICH process, November 2011. At *Step 4* of the process, the final draft is recommended for adoption to the regulatory bodies of the European Union, Japan, and the United States.

[2] Arabic numbers reflect the organizational breakdown of the document endorsed by the ICH Steering Committee at Step 4 of the ICH process, November 2011.

guidance are different from the OECD or the IWGT recommendations, and are noted in the text. The following notes for guidance should be applied in conjunction with other ICH guidances.

C. Scope of the Guidance (1.3)

The focus of this guidance is testing of new *small molecule* drug substances, and the guidance does not apply to biologics. Advice on the timing of the studies relative to clinical development is provided in the ICH guidance *M3(R2) Nonclinical Safety Studies for the Conduct of Human Clinical Trials and Marketing Authorization for Pharmaceuticals* (ICH M3(R2) guidance).[3] The recommendations in the guidance should be applied in conjunction with other ICH guidances.

D. General Principles (1.4)

Genotoxicity tests can be defined as in vitro and in vivo tests designed to detect compounds that induce genetic damage by various mechanisms. These tests enable hazard identification with respect to damage to DNA and its fixation. Fixation of damage to DNA in the form of gene mutations, larger scale chromosomal damage, or recombination is generally considered to be essential for heritable effects and in the multistep process of malignancy, a complex process in which genetic changes might possibly play only a part. Numerical chromosome changes have also been associated with tumorigenesis and can indicate a potential for aneuploidy in germ cells. Compounds that are positive in tests that detect such kinds of damage have the potential to be human carcinogens and/or mutagens. Because the relationship between exposure to particular chemicals and carcinogenesis is established for humans, although a similar relationship has been difficult to prove for heritable diseases, genotoxicity tests have been used mainly for the prediction of carcinogenicity. Nevertheless, because germ line mutations are clearly associated with human disease, the suspicion that a compound might induce heritable effects is considered to be just as serious as the suspicion that a compound might induce cancer. In addition, the outcome of genotoxicity tests can be valuable for interpreting carcinogenicity studies.

II. THE STANDARD TEST BATTERY FOR GENOTOXICITY (2)

A. Rationale (2.1)

Registration of pharmaceuticals requires a comprehensive assessment of their genotoxic potential. Extensive reviews have shown that many compounds that are mutagenic in the bacterial reverse mutation (Ames) test are rodent carcinogens. Addition of in vitro mammalian tests increases sensitivity for detection of rodent carcinogens and broadens the spectrum of genetic events detected, but also decreases the specificity of prediction (i.e., increases the incidence of positive results that do not correlate with rodent carcinogenicity). Nevertheless, a battery approach is still reasonable because no single test is capable of detecting all genotoxic mechanisms relevant in tumorigenesis.

[3] We update guidances periodically. To make sure you have the most recent version of a guidance, check the FDA Drugs guidance Web page at *http://www.fda.gov/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/default.htm*.

The general features of a standard test battery are as follows:

i. Assessment of mutagenicity in a bacterial reverse gene mutation test. This test has been shown to detect relevant genetic changes and the majority of genotoxic rodent and human carcinogens.

ii. Genotoxicity should also be evaluated in mammalian cells in vitro and/or in vivo as follows.

Several in vitro mammalian cell systems are widely used and can be considered sufficiently validated: the in vitro metaphase chromosome aberration assay, the in vitro micronucleus assay (note 1) and the mouse lymphoma L5178Y cell Tk (thymidine kinase) gene mutation assay (MLA). These three assays are currently considered equally appropriate and therefore interchangeable for measurement of chromosomal damage when used together with other genotoxicity tests in a standard battery for testing of pharmaceuticals, if the test protocols recommended in this guidance are used. In vivo test(s) are included in the test battery because some agents are mutagenic in vivo but not in vitro (note 2) and because it is desirable to include assays that account for such factors as absorption, distribution, metabolism, and excretion. The choice of an analysis either of micronuclei in erythrocytes (in blood or bone marrow), or of chromosome aberrations in metaphase cells in bone marrow, is currently included for this reason (note 3). Lymphocytes cultured from treated animals can also be used for cytogenetic analysis, although experience with such analyses is less widespread.

In vitro and in vivo tests that measure chromosomal aberrations in metaphase cells can detect a wide spectrum of changes in chromosomal integrity. Breakage of chromatids or chromosomes can result in micronucleus formation if an acentric fragment is produced; therefore, assays that detect either chromosomal aberrations or micronuclei are considered appropriate for detecting clastogens. 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. The MLA detects mutations in the Tk gene that result from both gene mutations and chromosome damage. There is some evidence that MLA can also detect chromosome loss.

There are several additional in vivo assays that can be used in the battery or as follow-up tests to develop weight of evidence in assessing results of in vitro or in vivo assays (see below). Negative results in appropriate in vivo assays (usually two), with adequate justification for the endpoints measured, and demonstration of exposure (see section IV.D (4.4)) are generally considered sufficient to demonstrate absence of significant genotoxic risk.

B. Description of the Two Options for the Standard Battery (2.2)

The following two options for the standard battery are considered equally suitable (see note 4):

Option 1

i. A test for gene mutation in bacteria.

ii. A cytogenetic test for chromosomal damage (the in vitro metaphase chromosome aberration test or in vitro micronucleus test), or an in vitro mouse lymphoma Tk gene mutation assay.

iii. An in vivo test for genotoxicity, generally a test for chromosomal damage using rodent hematopoietic cells, either for micronuclei or for chromosomal aberrations in metaphase cells.

Option 2

i. A test for gene mutation in bacteria.

ii. An in vivo assessment of genotoxicity with two different tissues, usually an assay for micronuclei using rodent hematopoietic cells and a second in vivo assay. Typically, this would be a DNA strand breakage assay in liver, unless otherwise justified (see below; also section IV.B (4.2) and note 12).

There is more historical experience with Option 1, partly because it is based on the ICH S2A guidance and the ICH S2B guidance. Nevertheless, the reasoning behind considering Options 1 and 2 equally suitable is as follows: When a positive result occurs in an in vitro mammalian cell assay, clearly negative results in two well-conducted in vivo assays, in appropriate tissues and with demonstrated adequate exposure, are considered sufficient evidence for lack of genotoxic potential in vivo (see section V.D.1.i (5.4.1.1) below). Thus, a test strategy in which two in vivo assays are conducted is the same strategy that should be used to follow up a positive result in vitro (see note 4).

Under both standard battery options, either acute or repeat-dose study designs in vivo can be used. In case of repeated administrations, attempts should be made to incorporate the genotoxicity endpoints into toxicity studies, if scientifically justified. When more than one endpoint is evaluated in vivo, it is preferable that they are incorporated into a single study. Often sufficient information on the likely suitability of the doses for the repeat-dose toxicology study is available before the study begins and can be used to determine whether an acute or an integrated test would be suitable.

For compounds that give negative results, the completion of either option of the standard test battery, performed and evaluated in accordance with current recommendations, will usually provide sufficient assurance of the absence of genotoxic activity and no additional tests are warranted. Compounds that give positive results in the standard test battery might, depending on their therapeutic use, need to be tested more extensively (see section V (5)).

There are several in vivo assays that can be used as the second part of the in vivo assessment under Option 2 (see section IV.B (4.2)), some of which can be integrated into repeat-dose toxicology studies. The liver is typically the preferred tissue because of exposure and metabolizing capacity, but choice of in vivo tissue and assay should be based on factors such as any knowledge of the potential mechanism, of the metabolism in vivo, or of the exposed tissues thought to be relevant.

Information on numerical changes can be derived from the mammalian cell assays in vitro and from the micronucleus assays in vitro or in vivo. Elements of the standard protocols that can indicate such potential are elevations in the mitotic index, polyploidy induction, and micronucleus evaluation. There is also experimental evidence that spindle poisons can be detected in MLA. The preferred in vivo cytogenetic test under Option 2 is the micronucleus assay, not a chromosome aberration assay, to include more direct capability for detection of chromosome loss (potential for aneuploidy).

The suggested standard set of tests does not imply that other genotoxicity tests are generally considered inadequate or inappropriate. Additional tests can be used for further investigation of genotoxicity test results obtained in the standard battery (see sections IV.B (4.2) and V (5)). Alternative species, including nonrodents, can also be used if indicated, and if sufficiently validated.

Under conditions in which one or more tests in the standard battery cannot be employed for technical reasons, alternative validated tests can serve as substitutes, provided sufficient scientific justification is given.

C. Modifications to the Test Battery (2.3)

The following sections describe situations where modification of the standard test battery might be advisable.

1. Exploratory Clinical Studies (2.3.1)

For certain exploratory clinical studies, fewer genotoxicity assays or different criteria for justification of the maximum dose in vivo might apply (see ICH M3(R2) guidance).

2. Testing Compounds That Are Toxic to Bacteria (2.3.2)

In cases where compounds are highly toxic to bacteria (e.g., some antibiotics), the bacterial reverse mutation (Ames) test should still be carried out, just as cytotoxic compounds are tested in mammalian cells, because mutagenicity can occur at lower, less toxic concentrations. In such cases, any one of the in vitro mammalian cell assays should also be done (i.e., Option 1 should be followed).

3. Compounds Bearing Structural Alerts for Genotoxic Activity (2.3.3)

Structurally alerting compounds (note 5) are usually detectable in the standard test battery since the majority of *structural alerts* are defined in relation to bacterial mutagenicity. A few chemical classes are known to be more easily detected in mammalian cell chromosome damage assays than bacterial mutation assays. Thus, negative results in either test battery with a compound that has a structural alert is usually considered sufficient assurance of a lack of genotoxicity. However, for compounds bearing certain specific structural alerts, modification to standard protocols can be appropriate (note 5). The choice of additional test(s) or protocol

modification(s) depends on the chemical nature, the known reactivity, and any metabolism data on the structurally alerting compound in question.

4. Limitations to the Use of In Vivo Tests (2.3.4)

There are compounds for which many in vivo tests (typically in bone marrow, blood, or liver) do not provide additional useful information. These include compounds for which data on toxicokinetics or pharmacokinetics indicate that the compounds are not systemically absorbed and therefore are not available to the target tissues. Examples of such compounds are some radioimaging agents, aluminum-based antacids, some compounds given by inhalation, and some dermally or other topically applied pharmaceuticals. In cases where a modification of the route of administration does not provide sufficient target tissue exposure, and no suitable genotoxicity assay is available in the most exposed tissue, it might be appropriate to base the evaluation only on in vitro testing. In some cases, evaluation of genotoxic effects at the site of contact can be warranted, although such assays have not yet been widely used (note 6).

D. Detection of Germ Cell Mutagens (2.4)

Results of comparative studies have shown that, in a qualitative sense, most germ cell mutagens are likely to be detected as genotoxic in somatic cell tests so that negative results of in vivo somatic cell genotoxicity tests generally indicate the absence of germ cell effects.

III. RECOMMENDATIONS FOR IN VITRO TESTS (3)

A. Test Repetition and Interpretation (3.1)

Reproducibility of experimental results is an essential component of research involving novel methods or unexpected findings; however, the routine testing of drugs with standard, widely used genotoxicity tests often does not call for replication. These tests are sufficiently well-characterized and have sufficient internal controls that repetition of a clearly positive or negative assay is not usually warranted. Ideally, it should be possible to declare test results clearly negative or clearly positive. However, test results sometimes do not fit the predetermined criteria for a positive or negative call and therefore are declared *equivocal*. The application of statistical methods can aid in data interpretation; however, adequate biological interpretation is of critical importance. An equivocal test that is repeated might result in (1) a clearly positive outcome, and thus an overall positive result; (2) a negative outcome, so that the result is not reproducible and overall negative, or (3) another equivocal result, with a final conclusion that remains equivocal.

B. Recommended Protocol for the Bacterial Mutation Assay (3.2)

Advice on the protocols is given in the OECD guidelines (1997) and the IWGT report (Gatehouse et al., 1994).

1. Selection of Top Dose Level (3.2.1)

Maximum dose level

The maximum dose level recommended is 5000 micrograms (µg)/plate (or 5 microliters (µL)/plate for liquid test substance) when not limited by solubility or cytotoxicity.

Limit of solubility

For bacterial cultures, precipitating doses are scored, provided precipitate does not interfere with scoring, toxicity is not limiting the evaluation of the assay, and the top concentration does not exceed 5000 µg/plate (or 5 µL/plate for liquid test substance). If no cytotoxicity is observed, then the lowest precipitating dose should be used as the top dose scored. If dose-related cytotoxicity or mutagenicity is noted, irrespective of solubility, the top dose scored should be based on cytotoxicity as described below.

Limit of cytotoxicity

In the Ames test, the doses scored should show evidence of significant toxicity, but without exceeding a top dose of 5000 µg/plate. Toxicity might be detected by a reduction in the number of revertants, and/or clearing or diminution of the background lawn.

2. Study Design/Test Protocol (3.2.2)

The recommended set of bacterial strains (OECD) includes those that detect base substitution and frameshift mutations as follows:

- *Salmonella typhimurium* TA98
- *Salmonella typhimurium* TA100
- *Salmonella typhimurium* TA1535
- *Salmonella typhimurium* TA1537 or TA97 or TA97a
- *Salmonella typhimurium* TA102 or *Escherichia coli* WP2 *uvrA* or *Escherichia coli* WP2 *uvrA* (pKM101)

One difference from the OECD and the IWGT recommendations is that based on experience with testing pharmaceuticals, a single bacterial mutation (Ames) test is considered sufficient when it is clearly negative or positive, and is carried out with a fully adequate protocol, including all strains with and without metabolic activation, a suitable dose range that fulfills criteria for top dose selection, and appropriate positive and negative controls. Also, for testing pharmaceuticals, either the plate incorporation or the pre-incubation method is considered appropriate for this single experiment (note 7). Equivocal or weak positive results might indicate that it would be appropriate to repeat the test, possibly with a modified protocol such as appropriate spacing of dose levels.

C. Recommended Protocols for the Mammalian Cell Assays (3.3)

Advice on the protocols is given in the OECD guidelines (1997) and the IWGT publications (e.g., Kirsch-Volders et al., 2003; Moore et al., 2006); advice on interpreting MLA results is also

given (Moore et al., 2006), including use of a global evaluation factor. Several differences from these recommendations are noted here for testing pharmaceuticals, notably for selection of the top concentration (see details below).

1. Selection of Top Concentration (3.3.1)

Maximum concentration

The maximum top concentration recommended is 1 millimolar (mM) or 0.5 milligram (mg)/milliliter (mL), whichever is lower, when not limited by solubility in solvent or culture medium or by cytotoxicity (note 8).

Limit of solubility

When solubility is limiting the ability to achieve the maximal concentration, the maximum concentration, if not limited by cytotoxicity, should be the lowest concentration at which minimal precipitate is visible in cultures, provided there is no interference with scoring. Evaluation of precipitation can be done by naked eye or by methods such as light microscopy, noting precipitate that persists or appears during culture (by the end of treatment).

Cytotoxicity

For in vitro cytogenetic assays for metaphase chromosome aberrations or for micronuclei, cytotoxicity should not exceed a reduction of about 50 percent in cell growth (notes 9 and 10). For the MLA, at the top dose there should be 80 to 90 percent cytotoxicity as measured by a relative total growth (RTG) between 20 to 10 percent (note 9).

2. Study Design/Test Protocols (3.3.2)

For the cytogenetic evaluation of chromosomal damage in metaphase cells in vitro, the test protocol should include the conduct of tests with and without metabolic activation, with appropriate positive and negative controls. Treatment with the test articles should be for 3 to 6 hours with a sampling time approximately 1.5 normal cell cycles from the beginning of the treatment. A continuous treatment without metabolic activation up to the sampling time of approximately 1.5 normal cell cycles should be conducted in case of negative or equivocal results for both short treatments, with and without metabolic activation. The same principles apply to the in vitro micronucleus assay, except that the sampling time is typically 1.5 to 2 normal cell cycles from the beginning of treatment to allow cells to complete mitosis and enter the next interphase. For both in vitro cytogenetic assays, there might be a need to modify the protocol for certain types of chemicals that could be more readily detected by longer treatment, delayed sampling times, or recovery periods (e.g., some nucleoside analogues and some nitrosamines). In the metaphase aberration assay, information on the ploidy status should be obtained by recording the incidence of polyploid (including endoreduplicated) metaphases as a percentage of the number of metaphase cells. For MLA, the test protocol should include the conduct of tests with and without metabolic activation, with appropriate positive and negative controls, where the treatment with the test article is for 3 to 4 hours. A continuous treatment

without metabolic activation for approximately 24 hours should be conducted in case of a negative or equivocal result for both short treatments, with and without metabolic activation. A standard MLA should include (1) the incorporation of positive controls that induce mainly small colonies and (2) colony sizing for positive controls, solvent controls, and at least one positive test compound concentration (should any exist), including the culture that gave the greatest mutant frequency.

For mammalian cell assays in vitro, built-in confirmatory elements, such as those outlined above (e.g., different treatment lengths, tests with and without metabolic activation), should be used. Following such testing, further confirmatory testing in the case of clearly negative or positive test results is not usually warranted. Equivocal or weak positive results might call for repeating tests, possibly with a modified protocol such as appropriate spacing of the test concentrations.

3. Positive Controls (3.3.3)

Concurrent positive controls are important, but *in vitro* mammalian cell tests for genetic toxicity are sufficiently standardized that use of positive controls can generally be confined to a positive control with metabolic activation (when it is done concurrently with the non-activated test) to demonstrate the activity of the metabolic activation system and the responsiveness of the test system.

IV. RECOMMENDATIONS FOR IN VIVO TESTS (4)

A. Tests for the Detection of Chromosome Damage In Vivo (4.1)

Either the analysis of chromosomal aberrations or the measurement of micronucleated polychromatic erythrocytes in bone marrow cells in vivo is considered appropriate for the detection of clastogens. Both rats and mice are considered appropriate for use in the bone marrow micronucleus test. Micronuclei can also be measured in immature (e.g., polychromatic) erythrocytes in peripheral blood in the mouse, or in the newly formed reticulocytes in rat blood (note 3). Likewise, immature erythrocytes can be used from any other species that has shown an adequate sensitivity to detect clastogens/aneuploidy inducers in bone marrow or peripheral blood (note 3). Systems for automated analysis (image analysis and flow cytometry) can be used if appropriately validated (OECD, 1997; Hayashi et al., 2000; 2007). Chromosomal aberrations can also be analyzed in peripheral lymphocytes cultured from treated rodents (note 11).

B. Other In Vivo Genotoxicity Tests (4.2)

The same in vivo tests described as the second test in the standard battery (Option 2) can be used as follow-up tests to develop weight of evidence in assessing results of in vitro or in vivo assays (notes 11 and 12). Although the type of effect seen in vitro and any knowledge of the mechanism can help guide the choice of in vivo assay, investigation of chromosomal aberrations or of gene mutations in endogenous genes is not feasible with standard methods in most tissues. Although mutation can be measured in transgenes in rodents, this entails prolonged treatment (e.g., 28 days) to allow for mutation expression, fixation, and accumulation, especially in tissues with little cell division (see note 12). Thus the second in vivo assay will often evaluate a DNA

damage endpoint as a surrogate. Assays with the most published experience and advice on protocols include the DNA strand break assays, such as the single cell gel electrophoresis (*Comet*) assay and alkaline elution assay, the in vivo transgenic mouse mutation assays and DNA covalent binding assays (all of which can be applied in many tissues (note 12)), and the liver unscheduled DNA synthesis (UDS) assay.

C. Dose Selection for In Vivo Assays (4.3)

Typically, three dose levels are analyzed (Hayashi et al., 2007).

1. Short-term Studies (4.3.1)

For short-term (usually 1 to 3 administrations) studies, the top dose recommended for genotoxicity assays is a limit dose of 2000 mg/kilogram (kg), if this is tolerated, or a maximum tolerated dose defined (for example, for the micronucleus assay (OECD)) as the dose producing signs of toxicity such that higher dose levels, based on the same dosing regimen, would be expected to produce lethality. Similar recommendations have been made for the Comet assay (Hartmann et al., 2003) and transgenic mutation assay (Heddle et al., 2000). Suppression of bone marrow red blood cell production should also be taken into account in dose selection. Lower doses are generally spaced at approximately two- to three-fold intervals below this dose.

2. Multiple Administration Studies (4.3.2)

Option 1 Battery: When the in vivo genotoxicity test is integrated into a multiple administration toxicology study, the doses are generally considered appropriate when the toxicology study meets the criteria for an adequate study to support human clinical trials; this can differ from dose selection criteria in the OECD guidelines for the in vivo micronucleus assay. This applies when the in vitro mammalian cell test is negative (or *nonrelevant positive* (see section V (5))).

Follow-up studies or Option 2 battery: When carrying out follow-up studies to address any indication of genotoxicity, or when using Option 2 with no in vitro mammalian cell assay, several factors should be evaluated to determine whether the top dose is appropriate for genotoxicity evaluation. Any one of the criteria listed below is considered sufficient to demonstrate that the top dose in a toxicology study (typically in rats) is appropriate for micronucleus analysis and for other genotoxicity evaluation:

i. Maximum feasible dose (MFD) based on physicochemical properties of the drug in the vehicle (provided the MFD in that vehicle is similar to that achievable with acute administration; note 13).

ii. Limit dose of 1000 mg/kg for studies of 14 days or longer, if this is tolerated.

iii. Maximal possible exposure demonstrated either by reaching a plateau/saturation in exposure or by compound accumulation. In contrast, substantial reduction in exposure to parent drug with time (e.g., ≥ 50% reduction from initial exposure) can disqualify the study (unless a blood sample taken in the first few days is available). If

this is seen in one sex, generally the sex with reduced exposure would not be scored at the end of the study, unless there is enhanced exposure to a metabolite of interest.

iv. Top dose is ≥ 50 percent of the top dose that would be used for acute administration, i.e., close to the minimum lethal dose, if such acute data are available for other reasons. (The top dose for acute administration micronucleus tests is currently described in the OECD guidelines as the dose above which lethality would be expected; similar guidance is given (e.g., Hartmann et al., 2003) for other in vivo assays.)

Selection of a top dose based only on an exposure margin (multiple over clinical exposure) without toxicity is not considered sufficient justification.

3. Testing Compounds That Are Toxic for Blood or Marrow (4.3.3)

Many compounds that induce aneuploidy, such as potent spindle poisons, are detectable in in vivo micronucleus assays in bone marrow or blood only within a narrow range of doses approaching toxic doses. This is also true for some clastogens. If toxicological data indicate severe toxicity to the red blood cell lineage (e.g., marked suppression of PCEs (polychromatic erythrocytes) or reticulocytes), doses scored should be spaced not more than about two fold below the top, cytotoxic dose. If suitable doses are not included in a multiweek study, additional data that could contribute to the detection of aneugens and some toxic clastogens could be derived from any one of the following:

i. Early blood sampling (at 3 to 4 days) is advisable when there are marked increases in toxicity with increasing treatment time. For example, when blood or bone marrow is used for micronucleus measurement in a multiweek study (e.g., 28 days), and reticulocytes are scored, marked hematotoxicity can affect the ability to detect micronuclei (i.e., a dose that induces detectable increases in micronuclei after acute treatment might be too toxic to analyze after multiple treatments (Hamada et al., 2001)). The early sample can be used to provide assurance that clastogens and potential aneugens are detected (but see notes 14 and 15).

ii. An in vitro mammalian cell micronucleus assay.

iii. An acute bone marrow micronucleus assay.

D. Demonstration of Target Tissue Exposure for Negative In Vivo Test Results (4.4)

In vivo tests have an important role in genotoxicity test strategies. The value of in vivo results 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 when in vitro test(s) have shown convincing evidence of genotoxicity, or when no in vitro mammalian cell assay is used. Evidence of adequate exposure could include toxicity in the tissue in question, or toxicokinetic data as described in the following section.

1. When an In Vitro Genotoxicity Test Is Positive (or Not Done) (4.4.1)

Assessments of in vivo exposure should be made at the top dose or other relevant doses using the same species, strain, and dosing route used in the genotoxicity assay. When genotoxicity is measured in toxicology assays, exposure information is generally available as part of the toxicology assessment.

Demonstration of in vivo exposure should be made by any of the following measurements:

i. Cytotoxicity

   a. For cytogenetic assays: By obtaining a significant change in the proportion of immature erythrocytes among total erythrocytes in the tissue used (bone marrow or blood) 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.

   b. For other in vivo genotoxicity assays: Toxicity in the liver or tissue being assessed (e.g., by histopathological evaluation or blood biochemistry toxicity indicators).

ii. Exposure

   a. Measurement of drug-related material either in blood or plasma. The bone marrow is a well-perfused tissue, and levels of drug-related materials in blood or plasma are generally similar to those observed in bone marrow. The liver is expected to be exposed for drugs with systemic exposure regardless of the route of administration.

   b. Direct measurement of drug-related material in target tissue, or autoradiographic assessment of tissue exposure.

If systemic exposure is similar to or lower than expected clinical exposure, alternative strategies might be called for such as:

(i) Use of a different route of administration;
(ii) Use of a different species with higher exposure;
(iii) Use of a different tissue or assay (see section II.C.4 (2.3.4), Limitations to the Use of Standard In Vivo Tests).

When adequate exposure cannot be achieved (e.g., with compounds showing very poor target tissue availability), conventional in vivo genotoxicity tests have little value.

2. When In Vitro Genotoxicity Tests Are Negative (4.4.2)

If in vitro tests do not show genotoxic potential, in vivo (systemic) exposure can be assessed by any of the methods above, or can be assumed from the results of standard absorption, distribution, metabolism, and excretion (ADME) studies in rodents done for other purposes.

E. Sampling Times for In Vivo Assays (4.5)

Selection of the sampling time in the in vivo MN (micronucleus), chromosomal aberration, and UDS test should follow OECD (1997).

When micronucleus analysis is integrated into multiweek studies, sampling of blood or bone marrow can be done the day after the final administration (see recommendation for additional blood sampling time in section III.C.3 (3.3.3) above).

For other genotoxicity assays, sampling time should be selected as appropriate for the endpoint measured; for example, DNA damage/strand break measurements are usually made a few (e.g., 2 to 6) hours after the last administration for the multiple daily administration. In the case of single administration, two sampling times should be used: a few hours and 24 hours after the treatment.

In principle, studies of any length can be considered appropriate, provided the top dose/exposure is adequate.

F. Number of Animals Analyzed (4.6)

The number of animals analyzed is determined by current recommendations for the micronucleus assay (OECD) or other genotoxicity assays and generally does not include all the animals treated for a toxicology study. Animals used for genotoxicity analyses should be randomly selected from the group used for the toxicology study.

G. Use of Male/Female Rodents in In Vivo Genotoxicity Tests (4.7)

If sex-specific drugs are to be tested, then the assay can be done in the appropriate sex. In vivo tests with the acute protocol can generally be carried out in only one sex. For acute tests, both sexes should be considered only if any existing toxicity, metabolism, or exposure (Cmax (peak concentration) or AUC (area under the plasma concentration curve)) data indicate a toxicologically meaningful sex difference in the species being used. Otherwise, the use of males alone is considered appropriate for acute genotoxicity tests. When the genotoxicity test is integrated into a repeat-dose toxicology study in two sexes, samples can be collected from both sexes, but a single sex can be scored if there is no substantial sex difference evident in toxicity/metabolism. The dose levels for the sex(es) scored should meet the criteria for appropriate dose levels (sections IV.C.2 (4.3.2) and IV.C.3 (4.3.3)).

Similar principles can be applied for other established in vivo genotoxicity tests.

H. Route of Administration (4.8)

The route of administration is generally the expected clinical route (e.g., oral, intravenous, or subcutaneous) but can be modified if appropriate to obtain systemic exposure (e.g., for topically applied compounds (see section II.C.4 (2.3.4)).

I. Use of Positive Controls for In Vivo Studies (4.9)

For in vivo studies, 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 (note 16).

V. GUIDANCE ON EVALUATION OF TEST RESULTS AND ON FOLLOW-UP TEST STATEGIES (5)

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 are not expected to detect nongenotoxic carcinogens. Experimental conditions, such as the limited capability of the in vitro metabolic activation systems, can 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. On the other hand a positive result in any assay for genotoxicity does not always mean that the test compound poses a genotoxic/carcinogenic hazard to humans.

Although positive in vitro data could indicate intrinsic genotoxic properties of a drug, appropriate in vivo data determine the biological significance of these in vitro signals in most cases. Also, because there are several indirect mechanisms of genotoxicity that operate only above certain concentrations, it is possible to establish a safe level (threshold) for classes of drugs with evidence for such mechanisms (see section V.B (5.2) below; see also Müller and Kasper, 2000; Scott et al., 1991; Thybaud et al., 2007).

A. Assessment of Biological Relevance (5.1)

The recommendations below assume that the test has been conducted using appropriate conditions such as spacing of doses and levels of toxicity.

Small increases in apparent genotoxicity in vitro or in vivo should first be assessed for reproducibility and biological significance. Examples of results that are not considered biologically meaningful include:

i. Small increases that are statistically significant compared with the negative or solvent control values but are within the confidence intervals of the appropriate historical control values for the testing facility

ii. Weak/equivocal responses that are not reproducible

If either of the above conditions applies, the weight of evidence indicates a lack of genotoxic potential, the test is considered negative or the findings not biologically relevant, and no further testing is called for.

B. Evaluation of Results Obtained in In Vitro Tests (5.2)

In evaluating positive results, especially for the microbial mutagenicity test, the purity of the test compound should be considered to determine whether the positive result could be attributable to a contaminant.

1. Evaluation of Positive Results Obtained In Vitro in a Bacterial Mutation Assay (5.2.1)

Because positive results in the Ames test are thought to indicate DNA reactivity, extensive follow-up testing to assess the in vivo mutagenic and carcinogenic potential would be warranted to assess the potential risk for treatment of patients, unless justified by appropriate risk-benefit analysis.

There are some well-characterized examples of artifactual increases in colonies that are not truly revertants. These increases can occur due to contamination with amino acids (i.e., providing histidine for *Salmonella typhimurium* strains or tryptophan for *Escherichia coli* strains), so that the bacterial reversion assay is not suitable for testing a peptide that is likely to degrade. Certain cases exist where positive results in bacterial mutation assays might be shown not to indicate genotoxic potential in vivo in humans, for example, when bacterial-specific metabolism occurs, such as activation by bacterial nitroreductases.

2. Evaluation of Positive Results Obtained In Vitro in Mammalian Cell Assays (5.2.1)

Recommendations for assessing weight of evidence and follow-up testing for positive genotoxicity results are discussed in IWGT reports (e.g., Thybaud et al., 2007). In addition, the scientific literature gives a number of conditions that can lead to a positive in vitro result of questionable relevance. Therefore, any in vitro positive test result should be evaluated based on an assessment of the weight of evidence as indicated below. This list is not exhaustive, but is given as an aid to decision-making.

i. The conditions do not occur in vivo (pH; osmolality; precipitates).
(Note that the 1 mM limit avoids increases in osmolality, and that if the test compound alters pH, it is advisable to adjust pH to the normal pH of untreated cultures at the time of treatment).

ii. The effect occurs only at the most toxic concentrations.
-in the MLA increases, at ≥ 80 percent reduction in RTG
-for in vitro cytogenetic assays, when growth is suppressed by ≥ 50 percent

If any of the above conditions apply, the weight of evidence indicates a lack of genotoxic potential; the standard battery (Option 1) can be followed. Thus, a single in vivo test is considered sufficient.

3. Evaluation of In Vitro Negative Results (5.2.3)

For in vitro negative results, further testing should be considered in special cases, such as the following (the examples given are not exhaustive, but are given as an aid to decision-making): the structure or known metabolism of the compound indicates that standard techniques for in vitro metabolic activation (e.g., rodent liver S9) might be inadequate; the structure or known activity of the compound indicates that the use of other test methods/systems might be appropriate.

C. Evaluation of Results Obtained From In Vivo Tests (5.3)

In vivo tests have the advantage of taking into account absorption, distribution, and excretion, which are not factors in in vitro tests, but are potentially relevant to human use. In addition, metabolism is likely to be more relevant in vivo compared to the systems normally used in vitro. If the in vivo and in vitro results do not agree, then the difference should be considered/explained on a case-by-case basis (e.g., a difference in metabolism; rapid and efficient excretion of a compound in vivo).

In vivo genotoxicity tests also have the potential to give misleading positive results that do not indicate true genotoxicity. As examples:

(i) Increases in micronuclei can occur without administration of any genotoxic agent, due to disturbance in erythropoiesis (Tweats et al., 2007, I).

(ii) DNA adduct data should be interpreted in the light of the known background level of endogenous adducts.

(iii) Indirect, toxicity-related effects could influence the results of the DNA strand break assays (e.g., alkaline elution and Comet assays).

Thus, it is important to take into account all the toxicological and hematological findings when evaluating the genotoxicity data (note 15). Indirect effects related to toxicological changes could have a safety margin and might not be clinically relevant.

D. Follow-up Strategies for Positive Results (5.4)

1. Follow-up to Findings In Vitro in Mammalian Cell Tests (5.4.1)

The following discussion assumes negative results in the Ames bacterial mutation assay.

i. Mechanistic/in vivo follow-up (5.4.1.1)

When there is insufficient weight of evidence to indicate lack of relevance, recommended follow-up for positive mammalian cell assays would be to provide experimental evidence, either by additional in vitro studies (see item *a* below) **or** by carrying out two appropriate in vivo assays (see item *b* below), as follows:

a. Mechanistic information that contributes to a weight of evidence for a lack of relevant genotoxicity is often generated in vitro, for example evidence that a test compound that induces chromosome aberrations or mutations in the MLA is not a DNA damaging agent (e.g., other negative mutation/DNA damage tests in addition to the Ames test; structural considerations), or evidence for an indirect mechanism that might not be relevant in vivo or might have a threshold (e.g., inhibition of DNA synthesis; reactive oxygen species produced only at high concentrations) (Galloway et al., 1998; Scott et al., 1991; Müller and Kasper, 2000). Similar studies can be used to follow up a positive result in the in vitro micronucleus assay, or in this case, evidence can include a known mechanism that indicates chromosome loss/aneuploidy, or centromere staining experiments (note 17) that indicate chromosome loss. Polyploidy is a common finding in chromosome aberration assays in vitro. Although aneugens can induce polyploidy, polyploidy alone does not indicate aneugenic potential and can simply indicate cell cycle perturbation; it is also commonly associated with increasing cytotoxicity. If polyploidy, but no structural chromosome breakage, is seen in an in vitro assay, generally a negative in vivo micronucleus assay with assurance of appropriate exposure would provide sufficient assurance of lack of potential for aneuploidy induction.

   If the above mechanistic information and weight of evidence supports the lack of relevant genotoxicity, only a single in vivo test with appropriate evidence of exposure is called for to establish the lack of genotoxic activity. This is typically a cytogenetic assay, and the micronucleus assay in vivo is called for when following up potential for chromosome loss.

   If there is not sufficient weight of evidence or mechanistic information to rule out relevant genotoxic potential, two in vivo tests are generally called for, with appropriate endpoints and in appropriate tissues (usually two different tissues), and with an emphasis on obtaining sufficient exposure in the in vivo models.

Or

b. Two appropriate in vivo assays should be done, usually with different tissues, and with supporting demonstration of exposure.

In summary, negative results in appropriate in vivo assays, with adequate justification for the endpoints measured and demonstration of exposure (see section IV.D.1 (4.4.1)) are considered sufficient to demonstrate absence of significant genotoxic risk.

ii. Follow-up to an in vitro positive result that is dependent upon S9 activation (5.4.1.2)

When positive results are seen only in the presence of the S9 activation system, it should first be verified that metabolic activation is responsible and not some other difference in conditions (e.g., low or no serum in the S9 mix, compared with ≥ 10 percent serum in the non-activated incubations). The follow-up strategy is then aimed at determining the relevance of the results in vitro to conditions in vivo, and will generally focus on in vivo studies in liver (note 18).

2. Follow-up to a Positive In Vivo Micronucleus Assay (5.4.2)

If there is an increase in micronuclei in vivo, all the toxicological data should be evaluated to determine whether a nongenotoxic effect could be the cause or a contributing factor (note 15). If nonspecific effects of disturbed erythropoiesis or physiology (such as hypo/hyperthermia) are suspected, an in vivo assay for chromosome aberrations might be more appropriate. If a *real* increase is suspected, strategies should be used to demonstrate whether the increase is due to chromosome loss or chromosome breakage (note 17). There is evidence that aneuploidy induction (e.g., with spindle poisons) follows a nonlinear dose response. Thus, it might be possible to determine that there is a threshold exposure below which chromosome loss is not expected and to determine whether an appropriate safety margin exists compared with clinical exposure.

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.

E. Follow-up Genotoxicity Testing in Relation to Tumor Findings in a Carcinogenicity Bioassay (5.5)

Additional genotoxicity testing in appropriate models can be conducted for compounds that were negative in the standard test battery but which have shown increases in tumors in carcinogenicity bioassay(s) with insufficient evidence to establish a nongenotoxic mechanism. To help understand the mode of action, additional testing can include modified conditions for metabolic activation in in vitro tests or can include in vivo tests measuring genetic damage in target organs of tumor induction, such as DNA strand break assays (e.g., comet or alkaline elution assays), liver UDS test, DNA covalent binding (e.g., by ^{32}P-postlabeling), mutation induction in transgenes, or molecular characterization of genetic changes in tumor-related genes (Kasper et al., 2007).

VI. NOTES (6)

Note 1. The in vitro micronucleus assay has been widely evaluated in international collaborative studies (Kirsch-Volders et al., 2003), is validated by the European Center for the Validation of Alternative Methods (ECVAM) (Corvi et al., 2008), and is the subject of an OECD guideline 487 (2010).

Note 2. There is a small but significant number of genotoxic carcinogens that are reliably detected by the bone marrow tests for chromosomal damage but have yielded negative/weak/conflicting results in the in vitro tests outlined in the standard battery options. Carcinogens such as procarbazine, hydroquinone, urethane, and benzene fall into this category. Some other examples from a survey of companies are described by Tweats et al., 2007, II.

Note 3. In principle, micronuclei in hematopoietic cells can be evaluated in bone marrow from any species, and in blood from species that do not filter out circulating micronucleated erythrocytes in the spleen. In laboratory mice, micronuclei can be measured in polychromatic erythrocytes in blood, and mature (normochromatic) erythrocytes can be used when mice are treated continuously for about 4 weeks or more. Although rats rapidly remove micronucleated erythrocytes from the circulation, it has been established that micronucleus induction by a range of clastogens and aneugens can be detected in rat blood reticulocytes (Wakata et al., 1998; Hamada et al., 2001). Rat blood can be used for micronucleus analysis, provided methods are used to ensure analysis of the newly formed reticulocytes (Hayashi et al., 2007; MacGregor et al., 2006) and the sample size is sufficiently large to provide appropriate statistical sensitivity, given the lower micronucleus levels in rat blood than in bone marrow (Kissling et al., 2007). Whichever method is chosen, bone marrow or blood, automated or manual analysis, each laboratory should determine the appropriate minimum sample size to ensure that scoring error is maintained below the level of animal-to-animal variation.

Some experience is now available for micronucleus induction in the dog and rhesus monkey (Harper et al., 2007; Hotchkiss et al., 2008). One example where such alternative species might be useful would be in evaluation of a human metabolite that was not sufficiently represented in rodents but was formed in the dog or monkey.

Note 4. Although the two options in the battery are equally suitable, specific knowledge about an individual test compound can indicate that one option is preferable. For example, if systemic exposure in animal models is equal to or less than anticipated clinical exposure, in vitro assays should be employed: Option 1 (see also sections II.C.4 (2.3.4) and IV.D.1 (4.4.1)). On the other hand, Option 2, including a test in liver, is recommended in cases where short-lived reactive metabolites are expected to be generated in the liver.

Note 5. Certain structurally alerting molecular entities are recognized as being causally related to the carcinogenic and/or mutagenic potential of chemicals. Examples of structural alerts include alkylating electrophilic centers, unstable epoxides, aromatic amines, azo-structures, *N*-nitroso groups, and aromatic nitro-groups (Ashby and Paton, 1994). For some classes of compounds with specific structural alerts, it is established that specific protocol

modifications/additional tests are important for optimum detection of genotoxicity (e.g., molecules containing an azo-group, glycosides, compounds such as nitroimidazoles requiring nitroreduction for activation, compounds such as phenacetin requiring a different rodent S9 for metabolic activation).

Note 6. There is some experience with in vivo assays for micronucleus induction in skin and colon (Hayashi et al., 2007), and DNA damage assays in these tissues can also be an appropriate substitute.

Note 7. A few chemicals are more easily detectable either with plate-incorporation or with pre-incubation methods, though differences are typically quantitative rather than qualitative (Gatehouse et al., 1994). Experience in the pharmaceutical industry where drugs have been tested in both protocols has not resulted in different results for the two methods, and, in the IWGT report (Gatehouse et al., 1994), the examples of chemical classes listed as more easily detectable in the pre-incubation protocol are generally not pharmaceuticals and are positive in in vivo genotoxicity tests in liver. These include short chain aliphatic nitrosamines, divalent metals, aldehydes (e.g., formaldehyde, crotonaldehyde), azo dyes (e.g., butter yellow), pyrrolizidine alkaloids, allyl compounds (allyl isothiocyanate, allyl chloride), and nitro (aromatic, aliphatic) compounds.

Note 8. The rationale for a maximum concentration of 1 mM for in vitro mammalian cell assays includes the following: The test battery includes the Ames test and an in vivo assay. This battery optimizes the detection of genotoxic carcinogens without relying on any individual assay alone. There is a very low likelihood of compounds of concern (DNA damaging carcinogens) that are not detected in Ames test or in vivo genotoxicity assay, but are detectable in an in vitro mammalian assay only above 1 mM. Second, a limit of 1 mM maintains the element of hazard identification, being higher than clinical exposures to known pharmaceuticals, including those that concentrate in tissues (Goodman & Gilman, 2001), and is also higher than the levels generally achievable in preclinical studies in vivo. Certain drugs are known to require quite high clinical exposures for therapeutic effect, e.g., nucleoside analogs and some antibiotics. Although comparison of potency with existing drugs can be of interest to sponsors, perhaps even above the 1 mM limit, it is ultimately the in vivo tests that determine relevance for human safety. For pharmaceuticals with unusually low molecular weight (e.g., less than 200) higher test concentrations should be considered.

Note 9. Although some genotoxic carcinogens are not detectable in in vitro genotoxicity assays unless the concentrations tested induce some degree of cytotoxicity, DNA damaging agents are generally detectable with only moderate levels of toxicity (Greenwood et al., 2004). As cytotoxicity increases, mechanisms other than direct DNA damage by a compound or its metabolites can lead to *positive* results that are related to cytotoxicity and not genotoxicity. Such indirect induction of DNA damage secondary to damage to non-DNA targets is more likely to occur above a certain concentration threshold. The disruption of cellular processes is not expected to occur at lower, pharmacologically relevant concentrations.

In cytogenetic assays, even weak clastogens that are known to be carcinogens are positive without exceeding a 50 percent reduction in cell counts. On the other hand, compounds that are

not DNA damaging, mutagenic, or carcinogenic can induce chromosome breakage at toxic concentrations. For both in vitro cytogenetic assays, the chromosome aberration assay and the in vitro micronucleus assay, a limit of about 50 percent growth reduction is considered appropriate.

For cytogenetic assays in cell lines, measurement of cell population growth over time (by measuring the change in cell number during culture relative to control, e.g., by the method referred to as population doubling (PD (see note 10)), has been shown to be a useful measure of cytotoxicity, as it is known that cell numbers can underestimate toxicity. For lymphocyte cultures, an inhibition of proliferation not exceeding about 50 percent is considered sufficient; this can be measured by mitotic index (MI) for metaphase aberration assays and by an index based on cytokinesis block for in vitro micronucleus assays. In addition, for the in vitro micronucleus assay, since micronuclei are scored in the interphase subsequent to a mitotic division, it is important to verify that cells have progressed through the cell cycle. This can be done by use of cytochalasin B to allow nuclear division but not cell division, so that micronuclei can be scored in binucleate cells (the preferred method for lymphocytes). For cell lines, other methods to demonstrate cell proliferation, including cell population growth over time (PD) as described above, can be used (Kirsch-Volders et al., 2003).

For MLA, appropriate sensitivity is achieved by limiting the top concentration to one with close to 20 percent Relative Total Growth (RTG) (10 to 20%) both for soft agar and for microwell methods (Moore et al., 2002). Reviews of published data using the current criteria found very few chemicals that were positive in MLA only at concentrations with less than 20 percent RTG and that were rodent carcinogens, and convincing evidence of genotoxic carcinogenesis for this category is lacking. The consensus is that caution is appropriate in interpreting results when increases in mutation are seen only below 20 percent RTG, and a result would not be considered positive if the increase in mutant fraction occurred only at ≤ 10 percent RTG.

In conclusion, caution is appropriate in interpreting positive results obtained as reduction in growth/survival approaches or exceeds 50 percent for cytogenetics assays or 80 percent for MLA. It is acknowledged that the evaluation of cells treated at these levels of cytotoxicity/clonal survival can result in greater sensitivity but bears an increased risk of nonrelevant positive results. The battery approach for genotoxicity is designed to ensure appropriate sensitivity without relying on single in vitro mammalian cell tests at high cytotoxicity.

To obtain an appropriate toxicity range, a preliminary range-finding assay over a broad range of concentrations is useful, but in the genotoxicity assay it is often critical to use multiple concentrations that are spaced quite closely (less than two-fold dilutions). Extra concentrations can be tested but not all concentrations need be evaluated for genotoxicity. It is not intended that multiple experiments be carried out to reach exactly 50 percent reduction in growth, for example, or exactly 80 percent reduction in RTG.

Note 10. For in vitro cytogenetic assays, it is appropriate to use a measure of relative cell growth to assess toxicity because cell counts can underestimate toxicity (Greenwood et al., 2004). Using calculated population doublings (see glossary) to estimate the 50 percent growth reduction level, it was demonstrated that the frequency of positive results with compounds that are not mutagenic

or carcinogenic is reduced, while agents that act via direct interaction with DNA are reliably positive.

Note 11. In certain cases, it can be useful to examine chromosome aberrations at metaphase in lymphocytes cultured from test animals after one or more administrations of test compound, just as bone marrow metaphase cells can be used. Because circulating lymphocytes are not replicating, agents that require replication for their genotoxic effect (e.g., some nucleoside analogs) are not expected to be detected in this cell type. Because some lymphocytes are relatively long-lived, in principle there is the potential for accumulation of unrepaired DNA damage in vivo that would give rise to aberrations when the cells are stimulated to divide in vitro. The in vivo lymphocyte assay can be useful in following up indications of clastogenicity, but in general another tissue such as liver is a more informative supplement to the micronucleus assay in hematopoietic cells because exposure to drug and metabolite(s) is often higher in liver.

Note 12. The inclusion of a second in vivo assay in the battery is to provide assurance of lack of genotoxicity by use of a tissue that is well exposed to a drug and/or its metabolites; a small number of carcinogens that are considered genotoxic gave positive results in a test in liver but were negative in a cytogenetics assay in vivo in bone marrow. These examples likely reflect a lack of appropriate metabolic activity or lack of reactive intermediates delivered to the hematopoietic cells of the bone marrow.

Assays for DNA strand breaks, DNA adducts, and mutations in transgenes have the advantage that they can be applied in many tissues. Internationally agreed protocols are not yet in place for all the in vivo assays, although considerable experience and published data and protocol recommendations exist for DNA strand break assays (Comet and alkaline elution assays), DNA adduct (covalent binding) measurements, and transgenic rodent mutation assays, in addition to the UDS assay. For a compound that is positive in vitro in the MLA and induces predominantly large colonies, and is also shown not to induce chromosome breakage in an in vitro metaphase assay, an in vivo assay for mutation, such as a transgenic mouse mutation assay, should be considered in preference to a DNA strand break assay. The UDS assay is considered useful mainly for compounds that induce bulky DNA adducts or are positive in the Ames test. Because cytotoxicity induces DNA strand breakage, careful cytotoxicity assessment is needed to avoid confounding the results of DNA strand break assays. This has been well-characterized for the in vitro alkaline elution test (Storer et al., 1996) but not yet fully validated for the Comet assay. In principle, the DNA strand break assays can be used in repeat-dose toxicology assays with appropriate dose levels and sampling times.

Because liver of mature animals is not a highly mitotic tissue, often a non-cytogenetic endpoint is used for the second assay; but when dividing hepatocytes are present, such as after partial hepatectomy, or in young rats (Hayashi et al., 2007), micronucleus analysis in liver is possible, and detects known genotoxic compounds.

Note 13. For common vehicles like aqueous methyl cellulose, this would usually be appropriate, but for vehicles such as Tween 80, the volume that can be administered could be as much as 30 fold lower than that given acutely.

Note 14. Caution is appropriate if the toxicological study design includes additional blood sampling, e.g., for measurement of exposure. Such bleeding could perturb the results of micronucleus analysis since erythropoiesis stimulated by bleeding can lead to increases in micronucleated erythrocytes.

Note 15. Increases in micronuclei can occur without administration of any genotoxic agent, due to disturbance in erythropoiesis (such as regenerative anemia; extramedullary hematopoiesis), stress, and hypo- and hyperthermia (reviewed by Tweats et al., 2007, I). In blood, changes in spleen function that affect clearance of micronucleated cells from the blood could lead to small increases in circulating micronucleated red blood cells.

Note 16. Positive controls for either short-term or repeat-dose genotoxicity studies:
For micronucleus (and other cytogenetic) assays, the purpose of the positive control is to verify that the individuals scoring the slides can reliably detect increases in micronuclei. This can be accomplished by use of samples from periodic studies (every few months) of small groups of animals (one sex) given acute treatment with a positive control. For manual scoring, such slides can be included in coded slides scored from each study. Positive control slides should not be obvious to readers based on their staining properties or micronucleus frequency. For automated scoring, appropriate quality control samples should be used with each assay.

For other in vivo genotoxicity assays, the purpose of positive controls is to demonstrate reliable detection of an increase in DNA damage/mutagenicity using the assay in the chosen species, tissue, and protocol. After a laboratory has demonstrated that it can consistently detect appropriate positive control compounds in multiple independent experiments, carrying out positive control experiments periodically is generally sufficient provided experimental conditions are not changed. However, currently it is considered that for the Comet assay, concurrent positive controls are advisable.

Note 17. Determination of whether micronucleus induction is due primarily to chromosome loss or to chromosome breakage could include staining micronuclei in vitro or in vivo to determine whether centromeres are present, e.g., using fluorescent in situ hybridization (FISH) with probes for DNA sequences in the centromeric region, or a labeled antibody to kinetochore proteins. If the majority of induced micronuclei are centromere positive, this suggests chromosome loss. (Note that even potent tubule poisons like colchicine and vinblastine do not produce 100% kinetochore positive micronuclei, but more typically 70 to 80 percent, and are accepted as primarily aneugens for assessing risk). An alternative approach is to carry out an in vitro or in vivo assay for metaphase structural aberrations; if negative, this would imply that micronucleus induction is related to chromosome loss.

Note 18. Standard induced S9 mix has higher activation capacity than human S9, and lacks phase two detoxification capability unless specific cofactors are supplied. Also, nonspecific activation can occur in vitro with high test substrate concentrations (see Kirkland et al., 2007). Genotoxicity testing with human S9 or other human-relevant activation systems can be helpful. Analysis of the metabolite profile in the genotoxicity test incubations for comparison with known metabolite profiles in preclinical species (in uninduced microsomes or hepatocytes, or in vivo) or in preparations from humans can also help determine the relevance of test results (Ku et

al., 2007), and follow-up studies will usually focus on in vivo testing in liver. A compound that gives positive results in vitro with S9 might not induce genotoxicity in vivo because the metabolite is not formed, is formed in very small quantities, or is metabolically detoxified or rapidly excreted, indicating a lack of risk in vivo.

VII. GLOSSARY (7)

Alkaline elution assay: See *DNA strand break assay.*

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 can lead to an altered protein.

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

Centromere/kinetochore: Structures in chromosomes essential for association of sister chromatids and for attachment of spindle fibers that move daughter chromosomes to the poles and ensure inclusion in daughter nuclei.

Clastogen: An agent that produces structural breakage of chromosomes, usually detectable by light microscopy.

Cloning efficiency: The efficiency of single cells to form clones. It is usually measured after seeding low numbers of cells in a suitable environment.

Comet assay: See *DNA strand break assay.*

Culture confluency: A quantification of the cell density in a culture by visual inspection.

Cytogenetic evaluation: Chromosome structure analysis in mitosis or meiosis by light microscopy or micronucleus analysis.

DNA adduct: Product of covalent binding of a chemical to DNA.

DNA repair: Reconstitution of the original DNA sequence after DNA damage.

DNA strand breaks: Single or double strand scissions in the DNA.

DNA strand break assay: Alkaline treatment that converts certain types of DNA lesions into strand breaks that can be detected by the alkaline elution technique, measuring migration rate through a filter, or by the single cell gel electrophoresis or Comet test (in which cells embedded in a thin layer of gel on a microscope slide are subjected to electric current, causing shorter pieces of DNA to migrate out of the nucleus into a *Comet tail*). The extent of DNA migration is measured visually under the microscope on stained cells.

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

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

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

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 nuclear DNA; it might contain a whole chromosome(s) or a broken centric or acentric part(s) of a chromosome(s).

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

Numerical chromosome changes: Chromosome numbers different from the original haploid or diploid set of chromosomes; for cell lines, chromosome numbers different from the modal chromosome set.

Plasmid: Genetic element in addition to the normal bacterial genome. A plasmid might be inserted into the host chromosome or form an extra-chromosomal element.

Point mutations: Changes in the genetic codes, 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 RNA.

Polyploidy: Numerical deviation of the modal number of chromosomes in a cell, with approximately whole multiples of the haploid number. Endoreduplication is a morphological form of polyploidy in which chromosome pairs are associated at metaphase as *diplochromosomes*.

Population doubling or culture growth: This can be calculated in different ways; one example of an appropriate formula is: Population doublings (PDs) = the log of the ratio of the final count (N) to the starting (baseline) count (Xo), divided by the log of 2. That is: $PD = [\log(N \div Xo)] \div \log 2$.

Recombination: Breakage and balanced or unbalanced rejoining of DNA.

RTG (relative total growth): This measure of cytotoxicity takes the relative suspension growth (based on cell loss and cell growth from the beginning of treatment to the second day post-treatment) and multiplies it by the relative plating efficiency at the time of cloning for mutant quantization.

Single cell gel electrophoresis assay: Comet assay. See *DNA strand break assay*.

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

Transgene: An exogenous or foreign gene inserted into the host genome, either into somatic cells or germ line cells.

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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