Guidance on a strategy for genotoxicity testing of chemicals

Stage 2: in vivo genotoxicity tests

Refer to the G-Strategy for an executive summary and introduction to this document.

Numbers in round brackets refer to references at the end of this document.

Overview of strategy

77. Stage 2 of the testing strategy involves an assessment of genotoxic activity in vivo in somatic tissues and in germ cells (when there is a need for the assessment of heritable effects and/or information on hazard classification of mutagens) (see Figure 3 in the main guidance document). The in vivo genotoxicity testing strategy has to be designed on a case-by-case basis and can be used to investigate aspects of in vivo mutagenicity, for example:

    - key end point(s) identified in Stage 1
    - genotoxicity in tumour target tissue(s)
    - potential for germ cell genotoxicity
    - in vivo genotoxic potential for chemicals which were negative in Stage 1 but where there is high or moderate and prolonged exposure
    - genotoxicity in site of contact tissues

78. It is thus possible for there to be one or more separate Stage 2 strategies designed to assess the above objectives for a particular test chemical. A revised in vivo Stage 2 strategy was presented in the previous COM guidance document (32) based on the selection of tests to provide information on one or more specific aspects such as species and/or tissue genotoxicity combined with investigation of particular genotoxic end points and modes of genotoxic action. This approach does not necessarily lead to the selection of the rodent BMMN test as the first assay. Furthermore, the rat liver UDS assay is no

longer recommended as a second assay (40 - discussed in paragraph 104, below). A table of in vivo genotoxicity tests and endpoints is provided in Annexe 1 (see the main guidance document).

79. Other factors that should be considered when determining an in vivo genotoxicity testing strategy include whether the testing strategy can be integrated into other regulatory toxicity tests (such as subacute or subchronic toxicity studies). Consideration needs to be given to the nature of the chemical (including physico-chemical properties), the results obtained from in vitro genotoxicity tests and the available information on the toxicokinetic and metabolic profile of the chemical (for example when selecting most appropriate species, tissue and end point). The routes of exposure in animal studies should be appropriate to ensure that the chemical reaches the target tissue. Routes unlikely to give rise to significant absorption in the test animal should therefore be avoided. Unless systemic exposure can be confirmed from other toxicological studies, or evident toxicity in the target organ is seen, or the intravenous route is used, confirmatory toxicokinetic studies to measure blood or tissue exposure as appropriate should be undertaken to accompany all in vivo genotoxicity studies to assess the adequacy of any negative results obtained (40).

80. The design of in vivo genotoxicity tests should incorporate appropriate approaches to reduce the number of animals used in tests, such as the integration of genotoxicity endpoints into repeat-dose studies, in line with the 3R's principle of Replacement, Reduction, Refinement. Options for reduction in animal usage include:

    - use of one sex only (if supported by metabolism data or other data indicating equivalence)
    - reduced numbers of sampling times for micronucleus and CA assays when repeat dosing is performed
    - combining micronucleus and comet assays into a single acute test employing repeated administrations of test chemical; integration of micronucleus and comet end points into repeat-dose toxicity (including transgenic mutation) studies, although it should be noted that the comet assay is difficult to integrate without using satellite groups (11, 127, 161).

It should also be possible to omit the concurrent positive control administrations in micronucleus, CA and transgenic rodent mutation assays (but not for the comet assay) where the test facility has appropriate historical positive control data (127) as long as positive control slides or tissues from positive control treated rodents 'banked' from previous treatments and coded in with the experimental samples, are included to demonstrate technical proficiency.

81. The toxic properties of test chemicals (such as acute toxicity, subchronic toxicity (including target organ effects), irritancy/corrosivity in contact with skin or mucous membranes), toxicokinetic and metabolism data will influence the choice of route of administration and the highest dose level achievable in in vivo mutagenicity tests. Dose selection for in vivo genotoxicity testing requires confirmation of the limit dose (LD) or estimation of the maximum tolerated dose (MTD), consideration of tissue-specific effects and in some instances (as discussed in paragraph 78, above), appropriate toxicokinetic data or toxicity data in the target tissue from other studies, to support tissue exposure to the chemicals and/or metabolites (40). OECD recommend the use of the LD in circumstances where "toxicity and solubility are not limiting factors, and if genetic toxicity is not expected based on data from structurally related substances". A LD of 2000 mg/kg bw/day for a treatment period of less than 14 days and 1000 mg/kg bw/day for a treatment period greater than 14 days are stated. In circumstances where toxicity is the limiting factor, OECD recommend use of the which is defined by OECD as "the highest dose that will be tolerated without evidence of study-limiting toxicity such that higher dose levels, based on the same dosing regimen, would be expected to produce lethality or evidence of pain, suffering or distress necessitating humane euthanasia" (108). It is possible that for some chemicals, the maximum dose may not be achievable (for example, due to solubility issues) and, in this case, the maximum feasible dose (MFD) may be applied.

82. The approach outlined for Stage 2 in Figure 3 (see the main guidance document) takes account of evidence to suggest that in vivo comet and rodent transgenic mutation assays have better sensitivity and specificity for the identification of rodent carcinogens compared with the rat liver UDS test, particularly for carcinogens that are negative in the in vivo micronucleus test (81). The initial in vivo genotoxicity testing strategy should

therefore involve selection of one or more of the core Stage 2 tests in rodents; namely, micronucleus tests (accompanied by specific modifications for aneuploidy if necessary), the transgenic gene mutation tests, or comet DNA damage assays in rodents. It is acceptable to undertake one in vivo genotoxicity test to investigate a specific end point identified from Stage 1 in vitro genotoxicity tests. In some instances, there may be a need to investigate more than one end point before reaching a full conclusion on in vivo genotoxic potential.

83. Stage 2 in vivo genotoxicity tests should be undertaken for test chemicals that are positive in any of the in vitro Stage 1 genotoxicity tests where there is a need to ascertain whether genotoxic activity can be expressed in vivo. There are many reasons why activity shown in vitro may not be observed in vivo (for example, lack of absorption, inability of the active metabolite to reach DNA, rapid detoxication and elimination). Data from in vivo genotoxicity tests is, therefore, essential before any definite conclusions can be drawn regarding the potential mutagenic or genotoxic hazard to humans from test chemicals which have given positive results in one or more in vitro genotoxicity tests. However, conclusions on mutagenic or genotoxic hazard and MoA may have to be derived from in vitro genotoxicity data for test chemicals when no in vivo genotoxicity testing is permitted.

84. In addition, an in vivo genotoxicity test may give positive results for chemicals which only act in vivo; experience though, has shown that such chemicals are rare (157, 158). Such agents include some kinase inhibitors, glucocorticoid receptor antagonists (65) and long-acting beta-2-agonists (132). In some instances positive results might be obtained from in vitro genotoxicity tests that are adapted to evaluate specific characteristics of the test chemical; for example, by the use of modified or non-standard exogenous metabolising fractions (106).

85. Positive results in any Stage 2 genotoxicity test should be assessed for an indication of a MoA and for evidence which may suggest a threshold of effect or irrelevant positive responses. The COM has previously discussed the relevance of high-dose only positives and recognises that these results may be secondary to non-genotoxic effects rather than being a genotoxic effect of the compound.

86. Examples of MoAs that may lead to irrelevant positive responses in micronucleus tests, include hypothermia or hyperthermia in rodents and compound induced increases in cell division of bone marrow erythroblasts (10, 143, 157). If the conclusion is reached that a relevant MoA occurs, then the chemical should be considered as an in vivo mutagen. MoA data will be important in considering whether a threshold or non-threshold approach to risk assessment can be used. The COM has published guidance on possible threshold modes of genotoxicity which can include:

    i) involvement of non-DNA targets, (for example, aneugen inhibition of microtubules)
    ii) the contribution of protective mechanisms (for example, repair of DNA adducts formed from many low molecular weight alkylating agents)
    iii) overload of detoxication pathways (for example, paracetamol)

87. Equivocal results may be resolved in some assays such as MNvit or CAvit by scoring more cells. In the absence of equivocal results or if there is a need to investigate specific mutagenic endpoints, tumour target organs, or the potential for heritable effects, supplementary in vivo genotoxicity tests should be undertaken (see Figure 3 in the main guidance document). This may involve repeating all or aspects of the initial Stage 2 testing strategy, or performing supplementary investigations (for example, mode of action investigations, such as DNA adducts or more specific germ cell testing) to investigate aspects of the genotoxicity of the test chemical which have not been resolved. There is a need to select the most appropriate test(s) on a case-by-case basis. All relevant factors, such as results from previous tests, and available information on toxicokinetics, toxicological effects and metabolism of the chemical, should be considered.

88. The development of testing strategies for germ cell mutagens is a rapidly evolving field. A summary of test methodologies that are currently under development and/or validation are outlined in the COM document ‘G7 Test Strategies for Germ Cell Mutagens’ (36).

89. One aspect of the approach to testing outlined in Figure 3 (see the main guidance document) is that hazard characterisation of germ cell genotoxicity can be included in the initial in vivo genotoxicity testing strategy if considered necessary. This is because

there are multi tissue in vivo genotoxicity assays (for example, transgenic rodent mutation assays and comet assay, though it should be noted that the standard comet assay has not been validated using mature sperm) which can also be used if a need to evaluate germ cell genotoxicity has been established. Additionally, germ cell mutation assays might be valuable on a case-by-case basis to provide information on heritable mutagenic effects, but these would form part of a supplementary in vivo genotoxicity testing strategy, if considered appropriate.

90. The COM reaffirms that a chemical considered a positive in vivo somatic cell mutagen should also be considered as a possible germ cell mutagen unless data can be provided to the contrary. The position held previously, that most if not all germ cell mutagens are also genotoxic in somatic cells, still holds true. It has been noted that some rare examples (for example, sodium orthovanadate, (7) where the mouse bone marrow micronucleus assay does not predict germ cell genotoxicity have been reported. However, the data on such compounds is conflicting and it is not known, for example, whether somatic mutations or DNA strand breaks would have been identified if other test systems (for example, transgenic assays and the comet assay) had been used and other tissues sampled (7, 19, 166).

91. It is plausible that other targets during the process of meiotic cell division may be unique to germ cells but not necessarily identical in both sexes (41). The COM evaluated advances in germ cell mutagenicity testing and some theories and hypotheses regarding human germ cell mutagenesis. It was concluded that it is not known whether unique germ cell mutagens exist (that is, chemicals that are germ cell mutagens but not somatic cell mutagens), but that this is partially because of the underutilisation of the currently accepted tests for assessing germ cell mutagenicity and a lack of investigations examining this. Recommended regimes for the analysis of mutations in germ cells are discussed fully in the COM document ‘G7 test strategies for germ cell mutagens’ (36).

Discussion of Stage 2 initial testing strategy: general aspects

92. There are many publications debating in vivo genotoxicity testing strategies. These include those developed by the GUM (German speaking section of the European Environmental Mutagen Society) which recommended a single study using a combined analysis for MN and comet induction in selected tissues (125), and those from the World Health Organization / International Programme on Chemical Safety (WHO / IPCS) which recommended cytogenetics (bone marrow) or gene mutation or alternative tests as defined by genotoxic endpoint, chemical class and reactivity (with consideration of factors such as bioavailability and metabolism) (37). ICH (2011), EFSA (2017) and ECHA (2017) have also proposed similar strategies to these. The in vivo genotoxicity testing strategy recommended by the COM acknowledges there can be a variety of reasons for undertaking in vivo genotoxicity tests and it is important to identify clearly the objective of the study and the critical aspects of in vivo genotoxicity to be addressed (as set out in the Overview of Stage 2 strategy) in order to develop a strategy accordingly, rather than simply specify preferred first and second tests. There is less data on the performance of in vivo genotoxicity assays for prediction of rodent carcinogenicity compared with data on the performance of in vitro genotoxicity tests. Transgenic rodent mutation assays and the in vivo micronucleus assay have been shown to exhibit complementarity regarding prediction of rodent carcinogenicity, consistent with the assessment of different mutagenic endpoints by these 2 assays (105). The IWGT has reported that an evaluation of 91 chemicals showed that TGR and in vivo comet assays have a similar ability to detect in vivo genotoxicity when tested with bacterial mutagens and Ames-positive carcinogens (85). Thus, genotoxic endpoint and MoA analysis of in vitro mutagenic activity is of considerable importance in helping to develop an initial in vivo genotoxicity testing strategy. The COM recommends that the initial in vivo genotoxicity testing strategy should be based on one or more tests selected from a relatively limited number of in vivo genotoxicity tests that have been specifically designed to provide the optimum amount of information on in vivo mutagenic potential of the test chemical. Where possible, consideration should be given to integrating in vivo genotoxicity testing into repeat-dose toxicity studies.

Discussion of Stage 2: recommended in vivo genotoxicity tests

93. Three recommended in vivo genotoxicity tests are outlined below and in Figure 2 (see the main guidance document). Information from one or more of these recommended core tests should provide sufficient in vivo genotoxicity data for most chemicals.

Rodent bone marrow and peripheral blood MN assay for clastogenicity and aneuploidy or rodent bone marrow CA assay for clastogenicity

94. The in vivo bone marrow or blood micronucleus (MNviv) assay is still the most widely used in vivo genotoxicity test (OECD TG 474: 'Mammalian Erythrocyte Micronucleus Test') (114). Most of the available in vivo data on the mutagenicity of chemicals have been obtained from studies using the MNviv test in bone marrow of mice. The bone marrow is readily accessible to chemicals that are present in the blood and a wide range of structurally diverse clastogens and aneugens has been detected using these methods. The use of peripheral blood is an alternative approach for both mice and rats (when the youngest fraction of reticulocytes is sampled) which provides equivalent data to the bone marrow assay and is technically less demanding. High throughput approaches to the peripheral blood MNviv utilising flow cytometry have been published (24, 155, 29) and the assay is well validated. The MNviv assay detects clastogenicity by measuring MN formed from acentric chromosome fragments in young (polychromatic) erythrocytes in the bone marrow or in reticulocytes of peripheral blood. It may also be used to identify the induction of chromosome loss. MN containing whole chromosomes (as opposed to fragments) can be identified with molecular kinetochore or centromeric labelling techniques. It should be noted that only aneuploidy produced by chromosome loss can be measured in the MNviv assay. The MNviv can be used in the initial in vivo genotoxicity strategy for generic testing for in vivo genotoxic potential and for assessment of clastogenicity and aneuploidy. Clastogenicity may be measured by

metaphase analysis of CA in bone marrow of rodents as an alternative approach to the use of the micronucleus assay.

95. Proposals have been published to incorporate micronucleus assays into routine rodent 28 day subacute toxicity studies following demonstration of the feasibility of such an approach (60, 87, 98). The evidence from one evaluation of micronucleus tests conducted on samples from short-term, subchronic and from a few chronic studies in mice has been published (167). In mice, MN in polychromatic erythrocytes represent DNA damage occurring in the last 72 hours, whilst MN in normochromatic erythrocytes represent average damage during the 30 day period prior to sampling (167).

96. The development of a simultaneous liver and peripheral blood micronucleus assay in adult rats has also been reported (150). A correlation between micronucleus induction in hepatocytes and hepatocarcinogenicity was shown and the authors proposed that the assay could detect micronucleus-inducing chemicals that require metabolic activation. Takasawa and others (2007), Suzuki and others (2009), and Hamada and others (2015) have also reported developments of an in vivo liver micronucleus assay, which has been discussed by IWGT (159, 160, 85), and it has been recommended that an OECD guideline should be developed.

Transgenic rodent (TGR) mutation assay for gene mutations

97. The transgenic rodent somatic and germ cell gene mutation assays (OECD TG 488, 116) can be used to assess gene mutations in a wide range of rodent tissues (including germ cells) using all routes of administration and is particularly valuable when investigating gene mutation as the genotoxic endpoint (84, 85). There is sufficient data to support the use of the Muta^{TM}mouse, BigBlue$^{®}$ mouse and rat (including use of λ cII transgene), LacZ plasmid mouse, and the gpt delta models in TG 488.

98. Molecular sequencing of induced mutations in transgenic targets can aid in interpretation of study results (particularly equivocal responses) and also provide mechanistic information. The OECD published a Detailed Review Paper (DRP) on Transgenic Rodent Gene Mutation Assays which led to the development of an OECD guideline that was adopted in July 2011, with revision in 2013 (OECD, 2013) and in 2020 (116). The

latest version focuses on updating recommended regimes for the analysis of mutations in germ cells (discussed fully in the COM document 'Test Strategies for Germ Cell Mutagens' (36). TG488 states that "when both somatic and germ cells need to be collected and/or tested, based on regulatory requirements, or toxicological information, a 28+28d regimen [that is, 28 days treatment with sampling 28 days following administration of the final dose] permits the testing of mutations in somatic tissues and tubule germ cells from the same animals" (100, Marchetti and others 2019).

Rodent alkaline Comet assay for DNA damage

99. The in vivo comet assay (OECD TG 489: In Vivo Mammalian Alkaline Comet Assay) (110) detects a wide spectrum of DNA damage including repairable DNA damage. A report of an international validation of the in vivo alkaline comet assay has been published (159) and formed the basis for the OECD guideline. An overview of the types of genetic lesions detected is given in paragraph 71 (see the main guidance document). The in vivo comet assay can detect chemicals that induce gene mutations and has produced positive results for nearly 90% of rodent carcinogens not detected by the rodent BMMN assay (81). It also shows high sensitivity when compared with TGR results in liver and the GI tract, and high sensitivity at detecting bacterial mutagens and mutagenic carcinogens (84, 85). Developments regarding the conduct of the in vivo alkaline comet assay were detailed in the previous COM guidance (COM, 2011). This assay can be used for elucidating positive in vitro genotoxicity findings and to evaluate genotoxicity in target organs of toxicity (63), however, it would not be an appropriate follow-up for a chemical causing aneuploidy in vitro. The comet assay can be applied to a wide range of species and in many tissues including site-of-contact tissues. In the absence of data indicating particular tissues of interest (for example, toxic findings or tissue accumulation seen in other studies), the IWGT concluded that comet analysis of the liver combined with the bone marrow or peripheral blood micronucleus assay will be sufficient in most cases. However, if systemic exposure is expected, or found, to be low then site-of-contact-effects in GI tract are effective (85). Validation of a protocol for a germ cell comet assay is ongoing.

100. The Committee considers that the in vivo comet assay has appropriate sensitivity to detect chemicals which induce both gene mutations and/or clastogenicity. Thus the in vivo comet assay is recommended as a core test in the initial in vivo genotoxicity testing strategy to assess DNA damage in multiple somatic tissues in a single study. It is possible to include the comet assay within other in vivo genotoxicity tests (161) or within standard subacute or subchronic regulatory toxicity tests (136), although the logistics of achieving the correct sampling time in relation to the final doses must be carefully considered (148).

Non-core in vivo test: rat liver UDS assay for DNA damage

101. The rodent liver UDS assay is an established approach for investigating genotoxic activity in the liver with the endpoint measured being indicative of DNA damage and subsequent repair in liver cells. The COM consideration of this assay and published evaluations now suggest it is less sensitive than the in vivo comet assay with regard to identification of genotoxicity in the liver. An analysis of the prediction of rodent carcinogens not identified by the micronucleus tests indicated that the comet assay was considerably better than the rat liver UDS assay at identifying rodent carcinogens (81, 148) Based on these analyses, EFSA concluded that the UDS assay was of limited usefulness in genotoxicity testing strategies, being only suitable for the detection of chemicals causing damage in the liver, and with a lower predictive value than the TGR and comet assays in detecting chemicals which cause gene mutations. For existing data sets, where the UDS assay has been used as a follow up to positive in vitro gene mutation findings, a UDS study is considered adequate only for positive results (40). The COM agree with this opinion and recommend use of the comet assay rather than rodent liver UDS in order to assess potential for DNA damage in vivo.
102. Another non-core test which is receiving increasing attention involves the detection of gene mutations at the endogenous phosphatidylinositol glycan complementation group A gene (Pig-A), a reporter gene in which mutations are currently detected in peripheral red blood cells of mammals (15, 29, 103). This assay has the potential advantage of being integrated into regulatory toxicity tests (28, 76) and it is noted that Pig-A mutations

increase with duration of dosing (103). The development of the assay was discussed by the IGWT (59) and it has since undergone validation in support of the development of an OECD TG (30, 117, 118).

Discussion of Stage 2: supplementary tests

103. Supplementary in vivo genotoxicity tests need to be considered on a case-by-case basis taking into account all relevant information. It is considered that for most chemicals, supplementary in vivo genotoxicity data should be unnecessary but on a case-by-case basis, specific aspects of MoA (for example, nature of DNA adducts) and further characterisation of germ cell genotoxicity (for example, characterisation of male and/or female germ cell clastogenicity including use of FISH, and the evaluation of heritable effects) may be required. DNA adduct studies can provide valuable information on potential genotoxicity as a follow up for in vitro mutagens which have yielded negative results in in vivo genotoxicity assays (130). DNA adduct data (including type of adduct, frequency, persistence, repair process) can be used to inform on MoA and its relationship to carcinogenesis, and should be considered in conjunction with other relevant data such as dosimetry, toxicity, genotoxicity and tumour data (73).
104. A brief outline of these additional Stage 2 methods is given in Table 1 below. Reference is also made in Table 1 to a number of tests for heritable genotoxic effects but it is noted that these tests, which involve the use of many animals and demand a high level of expertise, are comparatively rarely used. The COM is aware that there is the possibility that gender differences in germ cell mutagenesis may exist and this aspect may need to be considered on a case-by-case basis (41). The conclusions of COM's evaluation of germ cell testing methods are provided in a separate document (DOH, 2021).

Table 1. Supplementary in vivo genotoxicity tests

Assay	Endpoint	Guidance	Main attributes	Comments
Investigations of DNA Adducts				
^{32}P-postlabelling	DNA adducts	IWGT	Can be highly sensitive particularly with bulky adducts and if appropriate enrichment technique used.	Interpretation of results can be complex. Involves handling high-activity ^{32}P. (131)
Covalent binding to DNA A variety of methods can be used such as those involving radioactive decay measurements (for example, ^{14}C-) or isotope measurements (for example, Accelerator Mass Spectrometry AMS)	DNA Adducts	IWGT	Some methods (AMS) are potentially very sensitive and can provide data on DNA binding at levels of exposure similar to low level environmental exposures	Uses radiolabelled compound (very small amounts (for example, nanograms) in the case of AMS). Interpretation of results can be complicated (for example, by non-specific binding). (68)
Supplementary investigations of germ cell mutagenicity				
Analysis for clastogenicity/ aneuploidy	Structural and numerical changes in spermatogonia, spermatocytes or oocytes	OECD	Can provide information on nature of effects in spermatogonia, spermatocytes and/or oocytes of mice or rats	Can provide useful information on MoA. (138)
Spermatid micronucleus assay	Chromosomal aberrations and or lagging chromosomes	None available	Provides information of clastogenic and/or aneugenic effects in spermatocytes.	(5)

Assay	Endpoint	Guidance	Main attributes	Comments
Dominant lethal assay	Chromosomal/gene mutations	OECD	Provides information on unstable chromosomal changes in gametes that lead to fetal death after fertilization and can determine stage(s) of gametogenesis affected	Little used. needs relatively large numbers of animals (4)
Mouse specific locus test	Gene mutations	EPA	Provides information on genetic changes transmitted to the first generation progeny as basis for estimation of induced mutation frequency in humans	Very rarely used. Needs large numbers of animals (3)
Mouse heritable translocation test	Chromosomal changes	EPA	Provides information on chromosomal changes transmitted to the first generation progeny as basis for estimation of induced translocation frequency in humans	Very rarely used. Needs large numbers of animals (3)
Sperm Comet assay	Double strand breaks and/or apurinic sites in sperm head DNA	None available	Provides information on genetic instability in sperm	(156)
Spermatid UDS assay	Repair DNA synthesis in spermatocytes	EPA	Provides information on induction of DNA lesions	(146)

Summary stage 2 (in vivo genotoxicity testing)

105. The in vivo genotoxicity testing strategy has to be designed on a case-by-case basis and can be used to investigate aspects of in vivo mutagenicity, for example:

    - key end point(s) identified in Stage 1
    - genotoxicity in tumour target tissue(s)
    - potential for germ cell genotoxicity
    - in vivo genotoxic potential for chemicals which were negative in Stage 1 but where there is high or moderate and prolonged exposure
    - genotoxicity in site of contact tissues

106. The recommended in vivo genotoxicity test(s) include micronucleus assay, bone marrow cytogenetics, alkaline comet assay in rodents and transgenic rodent mutation assay. In some instances there may be a need to undertake more than one in vivo test to perform an initial assessment of in vivo genotoxic potential (for example, where endpoints cannot be assessed in one study and there is a need to investigate multiple endpoints before reaching conclusions on in vivo mutagenic potential). Multiple endpoints may be combined in a single study. If positive results are obtained it is important to consider the evidence for MoA and check the data for evidence of irrelevant positive results. Usually negative results obtained in a carefully selected in vivo test (possibly studying more than one endpoint and tissue) will be sufficient to address positive results found in vitro, provided that target tissue exposure is sufficient. However, a further test(s) may be needed if some of the genotoxic effects seen in Stage 1 in vitro tests have not been adequately studied in vivo (for example, the chemical affects multiple mutagenic endpoints), or other aspects of the genotoxic potential of the chemical had not been fully resolved (for example, in the case where an investigation of heritable effects was required). If equivocal results are obtained, then supplementary testing (including scoring of additional cells in the case of the comet and MN assays) may be needed. This may involve repeating some aspects of the recommended in vitro and/or in vivo genotoxicity tests or performing additional investigations (for example, MoA investigations, such as DNA adducts and/or more detailed consideration of heritable effects). The supplementary in vivo genotoxicity testing

strategy and selection of the most appropriate assays should be undertaken on a case-by-case basis. All relevant factors such as results from previous tests, structural alerts and available information on toxicokinetics, tissue toxicity and metabolism of the chemical, should be considered. In the absence of appropriate germ cell genotoxicity data, the COM considers it is reasonable to assume that all somatic cell mutagens have the potential to be germ cell mutagens.

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