Guidance on a strategy for genotoxicity testing of chemicals

Stage 1: in vitro genotoxicity testing

Please 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

34. The COM concluded in 1989, 2000 and 2011 that it was appropriate to concentrate on a relatively small number of assays, using validated, sensitive methods particularly chosen to avoid misleading negative or positive results when compared to in vivo testing results (51, 52, 79, 80, 101, 126). A detailed justification of the strategy is given in the previous version of the COM Guidance (32) and, as such, is not included here.

35. Misleading positive results are considered to be caused by a number of factors, including inappropriately high doses of chemical and the use of cell lines of rodent origin (for example, V79, CHO, CHL) that partially lack normal cell cycle control, have limited metabolic capacity (even with the addition of S9) and do not mimic site-specific metabolic capacity (135). The use of p53-competent human cells and careful control of cytotoxicity can help reduce the number of misleading positive results without compromising sensitivity (51, 52). The development of 3D tissue models is also hoped to reduce the number of misleading positive findings and improve the accuracy of predictions due to their improved metabolic capacity and proximity to in vivo gene expression and protein functions (6, 9, 128, 129). The current state of the science for 3D model development and validation is discussed in ‘G8 3D Tissue Models for Genotoxicity Testing’ (35).

36. As outlined above in paragraph 20 (please refer to the main guidance document) and shown in Figure 2, Stage 1 involves tests for genotoxic activity using in vitro methods

and comprises a 2 test core system, namely an in vitro bacterial test for gene mutation (Ames test) and an in vitro micronucleus test (MNvit), with the objective of assessing genotoxic potential by investigating 3 different end points (gene mutation, structural chromosomal damage and changes in chromosome number). A detailed justification of the strategy is given in the previous version of the COM Guidance (32).

37. A clear positive result in either of these 2 core tests is sufficient to define the chemical as an in vitro genotoxin, although further in vitro and/or in vivo testing may be undertaken to understand the relevance of the positive results. The committee considers this strategy allows for efficient identification of all genotoxic endpoints and that, by reducing the number of mammalian cell tests and following the most current version of the methodologies, the risk of misleading positive results (that is, when compared with in vivo genotoxicity data) is decreased.

38. Additional investigations of chemicals which give positive or repeated equivocal results in Stage 1 tests can include an assessment of mode(s) of in vitro genotoxic action. There are a number of reasons (discussed in paragraphs 43 to 45, below) why positive results in in vitro genotoxicity tests might occur by mode(s) of action not relevant to human health hazard assessment. Such MoA evaluation in vitro is particularly relevant for those chemicals (for example, cosmetics) where there is a regulatory constraint which precludes the use of in vivo genotoxicity assays in the testing strategy. The COM does not recommend the use of Stage 1 in vitro genotoxicity assays that have not been considered in detail in this guidance or for which OECD guidelines either do not exist or have been deleted. This includes assays for sister chromatid exchange, the in vitro unscheduled DNA synthesis (UDS) assay, the in vitro comet assay or tests using fungi or Drosophila. A table of genotoxic endpoints detected by each assay cited in Stage 1 of this strategy is given in Annexe 1.

39. For chemicals which give equivocal results or repeated small positive effects, when considering biological relevance, it is important to consider evidence of reproducibility in the same assay or in different assays detecting similar effects, and the magnitude of the induced genotoxic effect in relation to historical negative control data, and then consider

whether further in vitro genotoxicity testing is needed (64, 78). Further consideration of SAR data for these chemicals may also give valuable information (25).

40. If clear negative results are obtained in both core in vitro tests undertaken, it can generally be concluded that the chemical has no genotoxic activity. However, there are some occasions when additional in vitro and/or in vivo genotoxicity testing may be undertaken for chemicals giving a negative response in the 2 in vitro core genotoxicity tests. For example, in situations where tumours are found in rodents, where the in vitro metabolic activation systems are not optimal or where there are human-specific metabolites, there may be a need for further genotoxicity assessment. A further testing strategy would have to be designed on a case-by-case basis (79, 106). An IWGT working group has published guidance on this topic (75). An important part of any additional in vitro strategy should be consideration of the appropriate exogenous metabolic activation system (including alternative sources of S9 or other metabolic systems including genetically engineered cell lines) (88), or even the testing of specific, relevant metabolites. Further information on in vivo genotoxicity testing of such test chemicals is provided in Stage 2 of this strategy.

41. Information from other combinations of genotoxicity tests, which may include one or more non-core tests outlined below in paragraphs 66 to 71, may also give adequate data on all 3 endpoints on a case-by-case basis. In vitro genotoxicity tests (micronucleus scoring and comet) using human reconstructed skin may provide useful information on in vitro mutagenic hazard in circumstances where in vivo testing is not permitted, or when extensive dermal exposure is anticipated (for example, cosmetic ingredients) (2, 18, 137, 134).

42. The full Stage 1 strategy should be performed, and the results of studies evaluated before a decision is made on whether to proceed to Stage 2 testing or whether a conclusion on mutagenic hazard can be derived for test chemicals where no in vivo genotoxicity testing is permitted. An outline of Stage 0 and Stage 1 (in vitro genotoxicity testing) is given in Figure 2 and a description of the assays recommended is provided in the following paragraphs.

Discussion of Stage 1 tests: general aspects

43. The conduct of genotoxicity assays has improved over time and the overall sensitivity of in vitro testing strategies regarding prediction of rodent carcinogens is very high (126, 22).

44. Kirkland and others assessed the sensitivity of a combination of the Ames test and MNvit test to detect rodent carcinogens and in vivo genotoxicants (83). The authors stated that it is difficult to draw precise conclusions from the available sensitivity and specificity data since the databases of chemicals used vary. However, this data shows that mammalian cell genotoxicity tests can have low specificity and that combinations of in vitro genotoxicity tests result in high sensitivity for rodent carcinogens and in vivo genotoxicants. High sensitivity has always been a priority of genotoxicity testing strategies recommended by the COM (31, 32). COM evaluated the use of in vitro genotoxicity tests to predict rodent carcinogens and in vivo genotoxicants in June 2010 and concluded that there is no convincing evidence that any [relevant or DNA reactive] rodent carcinogen or in vivo genotoxicant would fail to be detected by using an in vitro genotoxicity test battery consisting of Ames and MNvit tests.

45. It is most likely that one of the few occasions where in vitro test strategies fail to detect mutagenic activity (that is, misleading negative results) could be explained by the absence of appropriate metabolic activity in vitro (12) or that the test chemical does not reach the cells. Approaches to resolving potential inadequacies in metabolic activation include structure based metabolism predictions, use of genetically modified target organisms (for example, CYP2E1 in Salmonella YG7108pin3ERb$_5$) (42), the use of exogenous metabolic activation systems derived from human sources, or recombinant human cytochrome P450 systems as an external activation system (88). Testing of isolated or synthesised metabolites may also be considered.

46. There are a number of MoAs by which a chemical may demonstrate an in vitro genotoxic effect that is either not relevant for humans (for example, a rat specific metabolite) or has

a threshold. The COM has reviewed the evidence for a number of threshold MoAs and published a general guidance statement in 2010.

47. Threshold MoAs can generally be attributable to non-DNA interactions or an overload of normal cellular physiology. In such cases a No Observed Effect Concentration (NOEC) can be determined and may be useful in evaluating risk. Investigations of a threshold-based MoA need to be designed on a case-by-case basis and can be complex to interpret (78).

48. There has been considerable debate regarding the highest concentration that should be used routinely in mammalian cell assays. The ICH has stated the maximum concentration tested for human pharmaceuticals should be 1 mM (or 500 µg/mL; whichever is lower) in mammalian cell genotoxicity assays when not limited by solubility in solvent or culture medium or by cytotoxicity. (72) This would have the effect of reducing the number of misleading positive results by avoiding the excessive concentrations where the cellular defence mechanisms might be overwhelmed (72). However, a reduction to 1 mM is not consistent with the OECD recommendation for a top concentration of 10 mM (or 2000 µg/mL, whichever is lowest) in mammalian cell genotoxicity assays, when not limited by solubility in solvent or culture medium or by cytotoxicity (109, 111, 112, 113). Morita and others (2014) showed that the reduction in the top concentration from 5000 to 2000 µg/mL for mammalian cell tests had no impact on sensitivity or specificity of in vitro chromosomal aberration tests. Another analysis of published data for the top concentration in mammalian cell genotoxicity tests identified a small number of carcinogens that (according to the publications) would not be detected in any part of a 3 test in vitro genotoxicity test battery (consisting of the Ames, mouse lymphoma and in vitro chromosomal aberration (CA) tests) if the testing concentration limit for mammalian cell assays were reduced from 10 mM to 1 mM (123). A further investigation of these carcinogens found that some positive results at concentrations above 1 mM were not reproducible (that is, they were not genotoxic in mammalian cells under current OECD guideline protocols) and others were positive at concentrations below 1 mM, particularly when continuous treatments in the absence of S-9 (not included in the original publications) were conducted. An upper limit for mammalian cells tests of

1 mM or 500 µg/ml (whichever is lower) has been proposed as sufficient to detect all genotoxic carcinogens that are negative in the Ames test (82). Several international organisations have updated their guidance regarding upper limit selection (for example, 72, 115, 54) although currently no international consensus has been reached. Precipitation of the chemical in the medium can also be used to define a maximal concentration or upper limit for testing. On balance, COM agreed that care should be taken to follow the appropriate guidance, depending on the chemical of interest.

49. There has also been considerable investigation of the role of excessive cytotoxicity in mammalian cells and choice of cell type as possible causes of misleading positive results (10, 43, 124, 126). The method used to assess cytotoxicity may affect the selection of the highest concentration tested and potentially the results obtained using mammalian cell genotoxicity assays (52, 80) and recommendations have been made to use cytotoxicity measures based on cell proliferation (53). However, it is important to note that although excessive cytotoxicity may lead to misleading positive results, it may also result in misleading negative results when pronounced cell cycle delay occurs. A similar conclusion was reached at an international symposium on regulatory aspects of genotoxicity testing (10).

50. Most cell lines used for genotoxicity testing lack appropriate metabolism leading to reliance on exogenous metabolic activation systems. These cell lines may often have impaired p53 function and altered DNA repair capacity (80). There is some evidence that human lymphocytes are less susceptible to misleading positives than the rodent cell lines currently used (for example, Chinese Hamster Ovary (CHO), V79, Chinese hamster lung (CHL)). The use of human cell lines HepG2, TK6 and MCL5 cells and the reconstructed human skin models and HepaRG have been evaluated (51, 80, 90). A brief summary of 3D models currently used for genotoxicity testing and those under development and/or validation has been prepared by COM (33).

51. The COM agrees that it is not necessary to undertake independent confirmatory in vitro tests when clear negative or positive results have been obtained provided the following criteria are satisfied:

    - there is no doubt as to the quality of the study design and the conduct of the test

- the spacing and range of test chemical concentrations rule out missing a positive response
- sufficient treatment conditions and sampling times have been used

52. It is recognised that it can be difficult to provide convincing evidence for absence of genotoxic effects. The investigator should consider the power of the study design and the past performance of the test system when formulating a protocol in order to optimise the chances of obtaining an unequivocal result from a single experiment and to ensure that any potential genotoxic effect is not missed.

53. There is a need to undertake further in vitro genotoxicity testing when an equivocal result is obtained (that is, neither clearly negative nor clearly positive by appropriate biological or statistical criteria). In the case of the MNvit and CAvit assays an equivocal result may be resolved by scoring more cells from the existing study (paragraph 87 in the main guidance document) and this should be assessed in the first instance. Additional genotoxicity tests need to be planned on a case-by-case basis and need not necessarily be undertaken in an identical fashion to the initial experiment(s). Indeed, it may be preferable to alter certain aspects of the study (for example, concentration levels investigated, treatment and sampling times, concentration of metabolic activation mix) to obtain supplementary data. It may also be appropriate to use a different genotoxicity test system, for example, a chromosomal aberration (CA) test, if there is equivocal evidence of clastogenicity from an in vitro micronucleus test, or an in vitro cell mutation assay (for example, TK or HPRT mutation assays) if there is equivocal evidence of gene mutations from an Ames test.

54. The use of historical negative control data to aid in the interpretation of genotoxicity test results has been considered particularly in relation to equivocal and small magnitude genotoxic effects (64). Advice has been published on approaches to collecting historical control data. Ideally data should be reported in terms of means and confidence intervals for the distribution of baseline genotoxic effects rather than observed ranges where outliers can have a disproportionate effect. The data set should be updated regularly and should be as large as possible. In addition, it is important that negative historical control data should have been generated using consistent methodology unless it can be

demonstrated that changes in protocol do not impact on the range of values reported in studies (64). In their most recent guidance OECD places an increased emphasis on the use of historical concurrent negative control data in the assessment of genotoxicity test results, including recommendations on how to build an historical control database (115).

55. If a chemical is considered on the basis of Stage 1 genotoxicity test results to have in vitro genotoxic potential but has not been tested in vivo, the COM considers it prudent to assume that the chemical may have in vivo genotoxic potential.

Discussion of Stage 1 strategy: specific core tests

In vitro bacterial tests for gene mutations

56. The most widely used in vitro mutagenicity test is the bacterial reverse mutation assay for gene mutations developed by Ames and his colleagues using Salmonella typhimurium (56) which forms the basis of OECD TG 471 (bacterial reverse mutation test). The very extensive database available for this assay justifies its inclusion in any initial genotoxicity testing for mutagenic hazard. Several strains of bacteria capable of detecting both base-pair and frame-shift mutations must be included, the validated strains being TA1535, TA1537 (or TA97 or TA97a), TA98 and TA100. These strains detect effects at G-C-rich sites. To detect certain oxidising mutagens or hydrazines, that produce effects at A-T-rich sites, an additional strain such as TA102 or a repair-deficient Escherichia coli strain (WP2uvrA or WP2uvrA (pKM101)) should be included. To detect cross-linking agents, it may be preferable to include TA102 or to add a repair proficient Escherichia coli strain (WP2 or WP2 (pKM101)). Testing should be carried out both in the presence and absence of an appropriate exogenous metabolic activation system such as induced rat liver S-9. Both plate-incorporation and pre-incubation methods are widely used, and either is acceptable in all test guidelines. There is ongoing consideration of the bacterial strains used. For example, the sensitivity and selectivity of the bacterial strains specified in OECD TG471 have been assessed (164) and the

current criteria for a valid Ames test and interpretation of test results have been evaluated (91).

57. Developments to the Ames test have been suggested to automate and minimise the amount of test chemical required; for example the Spiral Salmonella mutagenicity assay (20), Ames II™ test (49) and Ames MPF (50, 149). Whilst discussions at the OECD around assay developments are ongoing, the committee considers that these methods have not currently been developed to a point where they can be routinely used for regulatory submissions.

In vitro mammalian cell micronucleus assay (MNvit) for clastogenicity and aneuploidy

58. The COM recommends that equivalent information on clastogenicity could be obtained from the MNvit compared with CA testing in mammalian cells (metaphase analysis) but that aneuploidy could be more easily detected by MNvit. There have been extensive and authoritative investigations of the utility of the MNvit which have concluded that the MNvit is reliable and can be used as an alternative to the in vitro CA for the assessment of clastogenicity and has the benefit of more easily detecting aneuploidy (21). The MNvit is available as OECD TG 487 (In Vitro Mammalian Cell Micronucleus Test) (109).

59. The MNvit can be carried out in the absence or presence of cytochalasin B, which is used to block cytoplasmic division and generate binucleate cells (cytokinesis block methodology (CBMN)). The advantage of using cytochalasin B is that it allows clear identification that treated and control cells have divided in vitro during or after treatment and provides a simple assessment of cell proliferation. Moreover, a defined population of binucleate cells is available for scoring. In general, the use of cytochalasin B has no impact on the sensitivity of the test results (55, 94, 120, 162), however this is not the case for nanoparticles (34). In the absence of cytochalasin B, where all cells will be mononucleate, it is essential to have evidence that cells have divided.

60. MNvit protocol development and assay performance have been previously described (32, 51, 52). A flow cytometric approach to the micronucleus assay has also been developed (14). MNvit assay can be performed using most mammalian cell lines used in

genotoxicity testing however there is evidence that rodent cell lines with compromised p53 activity such as V79, CHO and CHL cells can give more misleading positive results than cell lines proficient for p53 activity such as TK6 and human lymphocytes (51). Overall, the COM's preference is for human lymphocytes which have a number of advantages over cell lines (for example, normal diploid primary human cells with some protection against oxidative damage when whole blood cultures are used). If cell lines are used, it is important that the cells have defined provenance (93) and that the impact of potential genetic drift of the cells cultured is understood (80). One particular area of protocol development that has been under considerable investigation is the most appropriate method(s) for estimating cytotoxicity. It has been suggested that using relative cell counts (RCC) may underestimate cytotoxicity, as proliferation is not measured, and lead to potentially misleading positive results (52). In addition, it should also be recognised that cytotoxicity may be underestimated when using vital stains as these also do not measure proliferation. In the absence of cytokinesis block, the relative increase in cell count (RICC) or relative population doubling (RPD) are comparable with replication index (RI) used with the cytokinesis block assay and are the most appropriate methods of cytotoxicity estimation. Consensus recommendations embedded in the OECD guideline 487 indicate that the target range for cytotoxicity in the MNvit is 55 ± 5%.

61. The MNvit assay in combination with the CB methodology and with pancentromeric or chromosome specific centromeric probes fluorescence in situ hybridisation (FISH) provides a sensitive assessment of cell proliferation and allows discrimination between chromosome breaks, chromosome loss (using pan-centromeric or anti-kinetochore antibodies) and chromosome non-disjunction and polyploidy (using chromosome-specific centromere probes) (86). It is therefore a useful model for assessing mode of action (122).

62. Binucleate cells obtained with the CBMN will usually be needed for determination of non-disjunction of chromosomes between daughter nuclei. Fenech has proposed that the CBMN assay can be further modified to provide comprehensive information on nucleoplasmic bridges (NPBs). This may provide information on chromosome

rearrangements or telomere end fusions, and nuclear buds (NBUDs) which may provide information on gene amplification (45, 46). Fenech proposed that the comprehensive CBMN assay should be considered as a 'cytome' method for measuring chromosomal instability and altered cellular viability (45). The 'cytome' method is complex and requires large amounts of blood and considerable technical skill. It is currently not suitable for routine testing of chemicals for genotoxicity but may provide useful information on MoA.

63. The flow-cytometry-based micronucleus assay (FCMMN) was developed to increase reproducibility and decrease turnaround time for the micronucleus test (89, 8). However, the modified assay did not overcome the potential issue of misleading positive results. A number of approaches were undertaken to overcome this and have been previously described (32). A separate approach to automation of the CBMN assay involves automated image analysis (27, 8, 139, 18, 95, 171, 17). This does provide some advantages over the FCMMN assay as the cells are not destroyed in the analysis and it can be applied to the cytokinesis blocked micronucleus assay. Thus, micronuclei can be scored in binucleated cells, cells containing multiple micronuclei can be easily identified and scored as a single event, and the image galleries and slides can be stored, allowing the experiment to be re-visited at a later date.

64. An interlaboratory evaluation of the MultiFlow DNA Damage kit— p53, gamma H2AX, Phospho-Histone H3 and polyploidy has been described by Bryce and others (16). This is a multiplexed in vitro genotoxicity assay based on flow cytometric analysis in which detergent-liberated nuclei are simultaneously stained with propidium iodide and labelled with fluorescent antibodies against p53, gH2AX, and phospho-histone H3. Polyploidy can be quantified as the proportion of 8n-positive events relative to the number of total events with 2n and greater DNA content.

65. From 7 laboratories assessing chemicals representing clastogens, aneugens and non-genotoxicants, with analysis based on global evaluation factors and using a multinomial logistic regression, assay sensitivity, specificity and concordance in relation to a priori MoA grouping were 92%. The authors suggest that the 2 distinct analysis strategies utilised can be used to rapidly and reliably predict a genotoxic MoA for new chemicals (16).

Discussion stage 1: non-core tests

In vitro chromosomal aberration assay in mammalian cells (metaphase analysis) for clastogenicity and aneuploidy

66. The in vitro CA assay in mammalian cells has been widely used in genotoxicity testing for many decades and provides information on chromatid and chromosome breaks, deletions and re-arrangements that are indicative of damage associated with adverse health outcomes. Only limited information can be obtained on potential aneugenicity by recording the incidence of polyploidy and/or modification of mitotic index (1). The COM notes that polyploidy may not be a reliable indicator for aneugenicity and may result from a number of different genetic changes (53, 102). It is possible to adapt the chromosome aberration assay to include the use of chromosome specific centromeric probes with fluorescence in situ hybridisation (FISH) to assess the potential for aneuploidy (99). An IWGT report (53) concluded that the preferred measure of cytotoxicity in the CA test should be one based on cell proliferation (for example, relative population doubling or relative increase in cell counts) compared to negative control cultures rather than simple cell counts. On balance it is considered preferable to use the in vitro micronucleus test for the initial assessment of clastogenic and aneugenic potential. The latest revision of the OECD test guideline (111) utilises a maximum test concentration corresponding to 10 mM (or 2 mg/mL) which is in-line with the revised MNvit assay (109).

In vitro mouse lymphoma assay for gene mutation and clastogenicity

67. The COM reaffirms the view stated in the 1989, 2000 and 2011 guidance, that the most appropriate in vitro mammalian cell gene mutation test is the mouse lymphoma assay. Protocol development and test data interpretation strategies were discussed previously (32).

68. A re-evaluation of published studies, many of which were undertaken by the US NTP, showed that a large number of these were uninterpretable or the outcomes equivocal (142). This assay is now described in a separate OECD TG ('Test 490: in vitro mammalian cell gene mutation test using the thymidine kinase gene') which was published in 2016 (112). Some authors have reported that the mouse lymphoma assay can detect, in addition to gene mutations and clastogenicity, information on recombination, deletion and aneuploidy (119, 145, 163). However, this has been contested from results showing that none of 7 reference aneugens were reliably detected at acceptable levels of cytotoxicity (54). It is possible that aneuploidy in these cells could be a secondary effect of chromosomal rearrangement. However, the COM considers that this assay does not reliably detect aneugens.

In vitro HPRT assays for gene mutation

69. An in vitro cell mutation assay which uses forward mutation in the hypoxanthine guanine phosphoribosyl transferase (HPRT) gene to assess mutations has been developed in several cell lines, principally CHO cells and is described in the revised OECD 476 guideline (113). TG476 recommends that the minimum number of cells required for the assay should allow for at least 10 spontaneous mutants being present in all phases of the test. The COM have previously considered the power of this assay and it was concluded that 10^7 surviving cells are required for a valid test, providing sufficient numbers of cells to maintain between 10 and 100 spontaneous mutations.

70. As discussed in paragraph 43, a number of research groups have developed genotoxicity assays based on MN measurement using commercial sources of human reconstructed skin (such as Episkin® and EpiDerm™) (18, 23, 48, 107, 137, 128, 129) or a co-culture technique involving reconstructed skin and mouse lymphoma L5178Y cells (48). Measurement of DNA damage using the comet assay in reconstructed skin has also been reported (126, 134, 129) and is considered to be sufficiently validated to start the OECD Test Guideline development process (129). The primary purpose in developing genotoxicity tests using reconstructed skin has been to supplement

genotoxicity data-packages for cosmetic chemicals where no in vivo genotoxicity tests are permitted.

In vitro alkaline Comet assay for DNA damage

71. The in vitro alkaline comet assay for DNA damage has been proposed as an alternative to clastogenicity assessment in mammalian cells since cell proliferation is not needed, therefore any cell type can be used (62, 165). The alkaline comet assay detects a wide range of genetic damage including single and double strand breaks, repair induced breaks, alkali labile lesions and abasic sites. There is evidence that the in vitro comet assay can be modified to detect DNA cross-linking agents (147). The comet-FISH assay has been developed to provide information on site specific DNA strand breaks (57, 133, 140). There is evidence that the in vivo comet assay can detect chemicals that induce gene mutations in vitro and in vivo (28, 81, 84, 85). Extrapolation from this suggests that the in vitro comet assay can also detect chemicals that induce gene mutations and this capability has been demonstrated (28). However, it is not recommended as a routine replacement for gene mutation tests in vitro. Thus, the comet assay measures DNA damage irrespective of genotoxic endpoint, with the exception of aneuploidy. A positive comet assay result may be due to repairable DNA damage or lesions which lead to cell death and not necessarily lead to mutations or MN. Negative results from an Ames test and MNvit would reduce the level of concern associated with positive results from an in vitro comet assay. Thus, the in vitro comet assay can serve as a useful adjunct to the recommended core-tests, especially in instances where in vivo testing is not permitted such as in cosmetics testing. Pfuhler and others (129) has reviewed the status of the development of the 3D organ-based models for genotoxicity testing. The authors concluded that the 3D skin comet assay was sufficiently validated to start the process of OECD test guideline development.

Summary stage 1 (in vitro genotoxicity testing)

72. The COM recommendations for Stage 1 testing remain the same as in the 2011 guidelines, namely that the 3 key endpoints of gene mutation, clastogenicity and aneuploidy can be detected by using 2 core in vitro tests. These should be undertaken according to the best international guidance available to avoid misleading positive or negative results. Data should be interpreted using appropriate statistical analysis and use of historical negative control data. It is important to note that the in vitro tests should be undertaken prior to any in vivo testing.

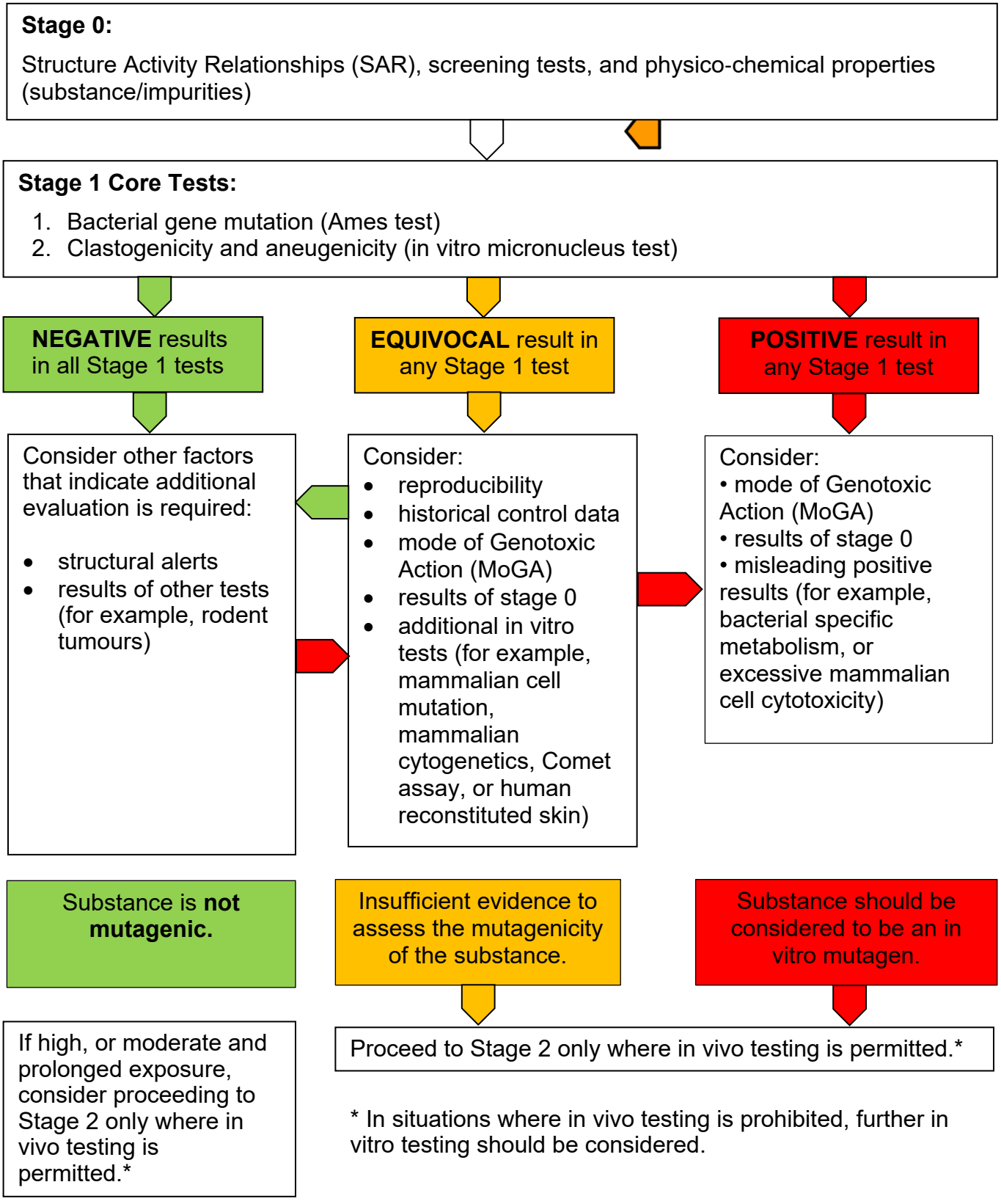
73. The COM confirms the need to understand MoA in order to derive conclusions regarding the biological importance of results. Data on MoA is important in elucidating whether genotoxicity tests give misleading negative or positive results, and also understanding of the MoA can help decisions with regard to devising a strategy for Stage 2 in vivo genotoxicity testing. There is a particular need to understand MoA for chemicals which cannot be subjected to in vivo genotoxicity tests (for example, cosmetics). In this particular instance, some useful additional information on genotoxicity may be provided by undertaking further testing, for example using in vitro mammalian cell gene mutation assays or in vitro MN and comet tests using reconstructed human skin.

74. The recommended 2 core genotoxicity tests in Stage 1 are the Ames test and MNvit. These recommended assays, when combined, provide sufficient information for the genotoxicity assessment of most chemicals and provide high sensitivity for the identification of rodent carcinogens and in vivo genotoxicants, and reduce the risk of misleading positive results when compared with a battery containing more than one mammalian cell test.

75. Results from non-core tests described in this document may provide useful additional information on in vitro mutagenic hazards on a case-by-case basis. In most instances misleading negative in vitro results are due to inadequate exogenous metabolic activation (88).

76. Some regulatory authorities may require an in vivo genotoxicity test where high, or moderate and prolonged, levels of exposure are expected (for example, most human medicines) in order to provide additional reassurance even when Stage 1 tests have given negative results. If a chemical is considered on the basis of Stage 1 test results to have in vitro mutagenic potential but has not been tested in vivo, the COM considers it prudent to assume that the chemical may have in vivo mutagenic potential.

Figure 2. Screening (Stage 0) and in vitro tests (Stage 1)



Accessible text version of Figure 2. Screening (Stage 0) and in vitro tests (Stage 1)

Stage 0:

Structure Activity Relationships (SAR), screening tests and physio-chemical properties (of substances and impurities).

Stage 1:

1. Bacterial gene mutation test (Ames test)
2. Clastogenicity and aneugenicity (in vitro micronucleus test)

Path 1: Negative results in all Stage 1 tests

Consider other factors that indicate additional evaluation is required:

- structural alerts,
- results of other tests (for example, rodent tumours)

Substance is not mutagenic. If high, or moderate and prolonged exposure, consider proceeding to Stage 2 only where in vivo testing is permitted (in situations where in vivo testing is prohibited, further in vitro testing should be considered)

OR

Consider other factors that indicate additional evaluation is required:

- structural alerts,
- results of other tests (for example, rodent tumours)

Consider:

- reproducibility
- historical control data
- mode of Genotoxic Action (MoGA)
- results of stage 0
- additional in vitro tests (for example, mammalian cell mutation, mammalian cytogenetics, Comet assay, or human reconstituted skin)

Insufficient evidence to assess the mutagenicity of the substance.

Proceed to Stage 2 only where in vivo testing is permitted (in situations where in vivo testing is prohibited, further in vitro testing should be considered).

Path 2: Equivocal result in any Stage 1 test

Consider:

- reproducibility

- historical control data
- mode of Genotoxic Action (MoGA)
- results of stage 0
- additional in vitro tests (for example, mammalian cell mutation, mammalian cytogenetics, Comet assay, or human reconstituted skin)

Consider other factors that indicate additional evaluation is required:

- structural alerts,
- results of other tests (for example, rodent tumours)

Substance is not mutagenic. If high, or moderate and prolonged exposure, consider proceeding to Stage 2 only where in vivo testing is permitted (in situations where in vivo testing is prohibited, further in vitro testing should be considered)

OR

Consider:

- reproducibility
- historical control data
- mode of Genotoxic Action (MoGA)
- results of stage 0
- additional in vitro tests (for example, mammalian cell mutation, mammalian cytogenetics, Comet assay, or human reconstituted skin)

Insufficient evidence to assess the mutagenicity of the substance.

Proceed to Stage 2 only where in vivo testing is permitted (in situations where in vivo testing is prohibited, further in vitro testing should be considered).

OR

Consider:

- reproducibility
- historical control data
- mode of Genotoxic Action (MoGA)
- results of stage 0
- additional in vitro tests (for example, Mammalian cell mutation, mammalian cytogenetics, Comet assay, or human reconstituted skin)

Consider:

- mode of Genotoxic Action (MoGA)
- results of stage 0

- misleading positive results (for example, bacterial specific metabolism, or excessive mammalian cell cytotoxicity)

Substance should be considered to be an in vitro mutagen.

Proceed to Stage 2 only where in vivo testing is permitted (in situations where in vivo testing is prohibited, further in vitro testing should be considered).

Path 3: Positive result in any Stage 1 test

Consider

- mode of Genotoxic Action (MoGA)
- results of stage 0
- misleading positive results (for example, bacterial specific metabolism, or excessive mammalian cell cytotoxicity)

Substance should be considered to be an in vitro mutagen.

Proceed to Stage 2 only where in vivo testing is permitted (in situations where in vivo testing is prohibited, further in vitro testing should be considered).

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Annexe 1

Tabulation of genotoxicity tests (in stages 1 and 2) and mutagenic/genotoxicity endpoints detected

Genotoxicity test	Mutagenic/genotoxicity endpoint detected	Comments
In vitro assays		
Ames	Gene mutation	Responds to wide range of DNA reactive mutagens when full set of *S. typhimurium* tester strains and E. coli with appropriate exogenous metabolic activation used.
Micronucleus test	Clastogenicity, aneuploidy	Centromere or kinetochore stains, with pancentromeric or chromosome specific centromeric probes using fluorescence in situ hybridisation (FISH) are required to distinguish between aneuploidy and clastogenicity.
Chromosomal aberrations	Clastogenicity, aneuploidy	Indications of aneuploidy from induction of polyploidy or increased mitotic index, but the use of chromosome specific centromeric probes fluorescence in situ hybridisation (FISH) required to assess the potential for aneuploidy. Very similar assay performance compared with micronucleus test
Mouse Lymphoma Assay	Gene mutation, clastogenicity	Distribution of large and small colony mutants can give information on induction of gene mutations versus clastogenicity. No convincing evidence that MLA can detect aneuploidy consistently.
Comet assay	DNA strand breaks and alkali labile sites	Can respond to a wide range of gene mutagens and clastogens but gives no information about modes of mutagenic action.
In vivo assays		
Rodent bone marrow or peripheral blood micronucleus assay	Clastogenicity, aneuploidy	A wide range of structurally diverse clastogens and aneugens have been detected. Distinguishing between clastogenic and aneugenic MoAs can be

Genotoxicity test	Mutagenic/genotoxicity endpoint detected	Comments
		investigated by use of centromere or kinetochore probes.
Rodent transgenic mutation assay	Gene mutations	Valuable for the investigation of gene mutation in a wide range of tissues including germ cells and particularly to confirm gene mutation as a mode of action.
Rodent comet assay	DNA strand breaks, alkali labile sites	Can respond to a wide range of gene mutagens and clastogens but gives no information about modes of mutagenic action. Does not detect aneugens. Valuable for detection of DNA damage in a wide range of tissues, but the standard alkaline assay not validated for mature sperm.
Rodent liver UDS	Unscheduled DNA synthesis	Endpoint measured is indicative of DNA damage and subsequent repair in liver cells, but now considered not as sensitive as other in vivo assays.
Pig-a gene mutation assay	Gene mutations	Endpoint measured is a reporter of gene mutation in rodents, but currently only extensively validated in blood cells.