

Review of Draft Document prepared by the Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM) Guidance on a strategy for genotoxicity testing and mutagenic hazard assessment of chemical substances

The Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM) has developed a testing strategy for assessment of genotoxicity for substances with pre existing genotoxicity/mutagenicity data for UK government departments and agencies with a human health mandate. While the COM is an expert advisory committee, and has no regulatory status, its documents, published in 1981, 1989 and 2000 are recognised internationally for their quality and scientific, forward thinking recommendations.

The strategy presented by COM is clearly presented and scientifically sound, and takes into consideration the current state of knowledge of mutagenicity and genotoxicity. Only parts of the strategy, as presented in Figures 1-3, would be applicable to the Bureau of Chemical Safety, HPFB at Health Canada.

The following points are considered of interest to Health Canada from a regulatory perspective:

1. The present document provides a testing strategy to mutagenicity assessment of chemicals with existing, but inadequate or incomplete genotoxicity data [Figure 1]. Specific *in vitro* tests are recommended [Figure 2, stage 0 and 1], and the need to conduct *in vivo* tests is assessed [Figure 3, Stage 2] to evaluate target organ genotoxicity *in vivo*. The core tests measure mutagenicity, some tests (eg comet) may give insight into mode of genotoxic activity. Considered in interpretation of test results were reproducibility, use of historical controls, potential for producing false positives, mode of genotoxic action (direct or indirect), structural alerts and results of other toxicity tests. The strategy is not a significant departure from Health Canada's mutagenicity guidelines (1992), but incorporates recent advances in test development and test result interpretation. In some cases, different genotoxicity tests are recommended.
2. For regulatory purposes, genotoxicity testing is conducted to identify mutagenic hazard, separate from carcinogenicity (Elespuru, 2009). The COM strategy also includes investigating genotoxicity in a tumour target tissue, potential germ cell genotoxicity and mutagenic endpoints identified from available genotoxicity data.[Figure 1]
3. No single assay can provide comprehensive information on all 3 levels of genetic damage (mutation, structural chromosome aberrations, numerical chromosomal aberrations). [Pg 6 line 12-23 point# 10] While comprehensive coverage of mutagenic potential could involve a range of assays employing a variety of organisms, target tissues including germ cells, from a regulatory point of view, requesting a large number of genotoxicity tests for mechanistic purposes would not be justified scientifically. [Pg 5 lines 8-31, pg 6 line 1-2; points# 7-8]
4. As mutagenicity is a subset of genotoxicity, the terms cannot be used interchangeably. In order to minimize confusion for the uninitiated reader, mutagenicity should be reserved for tests of the defined

endpoint (mutation, clastogenicity) that leads to permanent genetic change, as in describing the core tests [Statements 13-15]. However, if as was pointed out in the 2003 COM document on *in vivo* positive mutagens, aneuploidy may result from indirect DNA interaction via the spindle, then the *in vitro* micronucleus assay is not a mutagenic assay, but a genotoxicity assay. Then the description in the Figures indicating that the core tests measure mutagenic events is incorrect. Genotoxicity should be used when other forms of direct and indirect DNA interactions are included [Statement 8].

In earlier COM guidances (1989-2000), the emphasis was on avoiding missing positive findings (i.e. avoiding generating false negatives). The present document addresses 'misleading' false positives generated when mammalian cells *in vitro* are exposed to excessive doses of test chemicals. Reducing the top dose by up to 10-fold, regardless of chemical class, would not conform to OECD test guidelines. Chemicals that induce positive responses only at high doses, and chemicals with a higher physiological threshold for toxicity might not be detected (Parry, 2010; Muller, 2000) [pg 20 line 17-pg 21 line 13; point #43] While SAR information when available, could be used to assure that no potentially positive mutagenic or carcinogenic chemicals would be missed through a 10-fold reduction in the top dose (Parry, 2010), Health Canada toxicologists receive mutagenicity tests conducted on coded chemicals, with little or no identifying or structural information provided to the test facility.

As Elespuru (2009) pointed out, genotoxicity testing batteries are specifically designed to detect genotoxic substances. They are not designed to model the multifactorial process of carcinogenesis, nor predict carcinogenicity. Agents that are negative in rodent bioassays and positive in *in vitro* tests are not necessarily 'false positives' for mutagenicity. Mammalian *in vitro* genetic toxicity test results could not be used appropriately to extrapolate to *in vivo* systems.

6. The term 'threshold' was used in various contexts in the document. It should be clearly defined for its situations in the accompanying glossary. This term is also used in the regulatory context.

a) The concept of a threshold for toxicological concern (TTC) took into account differences in chemical class, and Munro described corresponding different thresholds. The level of 1.5 µg/kg bw/day was based on statistical analyses of carcinogen databases by Kroes, and may not be appropriate as a threshold exposure level for genotoxic chemicals, regardless of chemical class, since that would assume that all genotoxic chemicals were carcinogens. Rather, 1.5 µg/kg bw/day could represent a threshold exposure level for carcinogens, regardless of the mechanism that led to tumour induction, below which the risk for cancer would be negligible, regardless of chemical class. It may not be appropriate to suggested using the TTC exposure level to determine whether to follow-up positive *in vitro* genotoxicity test results.[pg 8, line 2-5, point #14; pg 10, line 4-19 point #20]

b) Food packaging guidelines drafted by Health Canada (2002) recommended that the extent of toxicity testing depend on potential human exposure level (levels of toxicological concern, LOC). An exposure threshold of 0.025 µg/kg bw/day was established, with genotoxicity testing at exposures exceeding 0.1 µg/kg bw/day.

c) A physiological threshold can be demonstrated when exposure to a toxic chemical is so high as to overwhelm normal cellular defence mechanisms, and produce apparent 'false' positive responses in mammalian cell genotoxicity tests *in vitro*, with genotoxic responses clearly negative over the lower dose ranges. Known mutagens produce linear, dose-related increases in genotoxicity assays at dose levels well below those that produce overt toxicity. Non-linear responses or positive responses only at toxic doses likely result from a non-genotoxic, threshold mechanism. [pg 20, line 17-pg 21, line 13, point #43] Excessive cytotoxicity from pronounced cell cycle delay may also lead to false negative results *in vitro*. [pg 21, line 21-22, point 44] Excessive toxicity *in vivo* may result in false positive findings due to overloading of detoxification pathways [pg 34, line 28, point #70]

7. Consideration of factors that affect mammalian cell tests *in vitro*, such as choice of cell line, culture medium, solvent and exogenous metabolic activation system should lead to improvements in the quality and interpretation of results from these studies [pg 21, line 14-pg 22, line 10; point #44].

8. The recommendation that the *in vitro* mammalian cell micronucleus test be used instead of the *in vitro* mammalian cell chromosomal aberration (metaphase analysis) test comes from the ability of the cytochalasin B protocol and FISH staining to detect aneuploidy. Also, metaphase analysis is more skills intensive, and probably more time consuming, but details concerning the nature of the translocations from metaphase analysis would not be detected in micronuclei. FISH staining led to significant improvements in the resolution of the metaphase test. The micronucleus test, has been validated (OECD guideline 487) and can detect aneugens as well as clastogens.[pg 24, line 3-22, point #51] Extensive details on preferred cell types and test protocols are presented in points 53-55 [pg 25-27]. The metaphase analysis test is the core test recommended by Health Canada for assessing clastogenic potential, although it does not detect aneugens. An analysis of performance of the micronucleus test and metaphase analysis showed slightly better specificity for metaphase analysis, while sensitivity was essentially identical [Kirkland 2010]

9. The mouse lymphoma test, even with colony size measurement and other protocol changes, is not recommended by COM. This assay is particularly susceptible to generating false positives and uninterpretable or equivocal results. [pg 28, line 3-23, points # 58-59]. Health Canada does not recommend this test except for information purposes.

10. The *in vitro* comet assay measures potentially repairable DNA damage, is not validated, is susceptible to producing false positives, and is not recommended by COM. [pg 29, line 4-29, part #61] The assay has not been used for regulatory purposes for food chemicals at Health Canada.

11. The rationale for selecting/conducting *in vivo* studies for mutagenic potential [Figure 3] includes identification of mutagenic endpoints from *in vitro* tests, tumour target tissues in carcinogenicity studies, potential for germ cell genotoxicity and high, moderate or sustained exposures. Tests recommended by COM include transgenic mutation assays, *in vivo* micronuclei or chromosomal aberrations and the *in vivo* comet assay. The micronuclei and chromosomal aberration assays are presently validated, and have been submitted to support food chemical petitions at Health Canada. *In*

vivo assays, like their *in vitro* counterparts, are susceptible to issues of overt toxicity, overloading detoxification pathways and possible threshold modes of genotoxicity, producing potentially false positive results. Micronuclei have been reportedly produced by chemicals that induce either hypothermia or hyperthermia, through stimulation of cell division in bone marrow erythroblasts. It is not clear how both conditions would give the same response, and what the time frame for the response following exposure would be [pg 34, line 13-28, point #70] Among other considerations, dose selection is based on maximum tolerated dose of test substance and/or its metabolites. A few chemicals are active only *in vivo*, due to factors including metabolic differences, influence of gut flora and pharmacological effects (folate depletion or receptor kinase inhibition), but it is not clear that exposures *in vivo* would be higher than *in vitro*, as it would be difficult to see how to extrapolate the exposures. [pg 33, lines 5-12, point #66]

12. The rodent bone marrow or peripheral blood micronucleus test for structural chromosomal damage is the standard *in vivo* mutagenicity test recommended by Health Canada for food chemicals including food packaging materials. COM recommends incorporating the *in vivo* micronucleus test into the enhanced protocol of the 28 day repeat dose rodent toxicity test. The 28 day rodent dietary study is also required by Health Canada for food packaging materials at 0.1 µg/kg bw/day (LOC 2).[pg 32, line 25, point# 65]

From the 2003 COM document on compounds positive in *in vivo* mutagenicity assay regarded as mammalian mutagens and hence potential genotoxic carcinogens, it was assumed that there were no threshold for their mutagenic activity unless appropriate mechanistic data identified a threshold related mechanism; e.g. induction of aneuploidy where site of initial action is not DNA but spindle apparatus. Such mechanistic data is rarely available. If aneuploidy results from indirect DNA interaction, then the *in vitro* micronucleus assay is not a mutagenic assay. When positive results in bone marrow assays were only seen at high dose levels associated with severe toxicity including lethality, the relevance of results was questioned, especially if the overall *in vivo* data, including carcinogenicity bioassays were negative. As micronuclei can be induced *in vivo* by mechanisms such as hypothermia, hyperthermia, stimulation of erythropoiesis and indirectly by severe cytotoxicity in the bone marrow, such positive results, if not supported by other *in vivo* data, may be regarded as artefacts (COM, 2003).

13. *In vivo* transgenic assays in rodents (TGR) detect mutations in bacteria isolated from a wide variety of rodent tissues, including germ cells. An OECD guideline is in preparation (G. Douglas, pers. Comm.). This assay will provide an *in vivo* gene mutation assay to confirm results from *in vitro* mammalian or bacterial gene mutations. Transgenic assays will also be used to assess target organ susceptibility, with molecular analysis of induced mutations providing mechanistic information. Health Canada scientists have been instrumental in the development of these assays, protocol modification and historical controls, and are preparing the draft OECD guideline. [pg 33, line 18-32; pg 37, line 14-32, pg 38, line 1-8; points #67, 77]

14. The *in vivo* Comet assay to detect DNA damage in non-proliferating tissues does not have a standard protocol, but is being recommended to replace the *in vivo/in vitro* rat liver UDS assay, presumably

because it can be used for cells from a variety of proliferating and non-proliferating tissues. Both assays detect potentially repairable DNA damage (except aneuploidy).[pg 31, line 3-30, point # 63; pg 32, line 3-30, points #64-65; pg 33, line 1-32, points #66-68]

15. Assessment of genotoxicity test results includes consideration of mode of genotoxic action, additional testing to fill in data gaps or resolve inconsistencies, and *in vivo* testing on a case-by-case basis to investigate target tissues in which tumours were induced in carcinogenicity studies. COM continues to consider a chemical positive in somatic cells *in vivo* as a possible germ cell mutagen, although the reverse may not necessarily follow.[pg 35, line 18-32, point #73; pg 36, line 3-10, point #74]

16. Numerous Structure Activity Relationship systems are available commercially [pg 11-15, points #24-29]. While an assessment of structure activity was conducted for 5 SAR programmes (DEREK, TOPKAT, MDL QSAR, MultiCASE and Toxtree) it is not easy to compare directly their relative sensitivity and specificity as presented in Annex 1[pg 45-46]. Health Canada has access to TOPKAT for concordance with positive bacterial mutations.

17. The use of screening tests for large numbers of chemicals would not be used in regulation. Commercial screening tests were assessed for sensitivity and specificity [pg 46] While high throughput tests could validate endpoints required, they are unlikely to replace mutagenicity tests, but may be useful for genotoxicity. [Pg 16 stage 0]

Additional comments:

Annex 1 Tabular information provided is useful to gain perspective on test performance, relationship between rodent carcinogens and *in vivo* genotoxins, endpoints detected and positive and negative features of the various *in vitro* and *in vivo* tests. These tables should be numbered.

Figure 2- Health Canada does not prohibit *in vivo* testing. It is unlikely that the human reconstructed skin system to test for micronuclei and the comet assay *in vitro* would be included for genotoxicity for food use chemicals.

Fig 3 *in vivo* assays. From a regulatory standpoint, mutagenic endpoints are preferred, with other tests considered as indicators of DNA interaction.

Glossary:

include: genotoxicity, mutagenicity (not just in text), MoGA (less trust little or no evidence) vs MOA (molecular based chemical genotoxicity to establish causality);
S9: should indicate derived from 9000xg supernatant
Topoisomerases: enzymes that ... also mention gyrase

TTC, and threshold should be defined and differentiated

References:

Consider adding the following references:

Elespuru RK, Agarwal R, Atrakchi AH, Bigger CAH, Heflich RH, Jagannath DR, Levy DD, Moore MM, Ouyang Y, Robison TW, Sotomayer RE, Cimino MC, Dearfield KL. (Current and future application of genetic toxicity assays: the role and value of in vitro mammalian assays Toxicological Sciences 109(2): 172-179. 2009)

Muller L, Sofuni T. (Appropriate levels of cytotoxicity for genotoxicity tests using mammalian cells in vitro Environmental and Molecular Mutagenesis 35: 202-205. 2000)

COM/03/55 November, 2003 Statement on guidance on considering high dose positive in vivo mutagenicity data in the bone marrow assays that may not be biologically significant with regard to considering a chemical to be an in vivo mutagen

For consistency, most journal titles were cited in full, so all should be, rather than abbreviated

The following typographical or editing problems were identified (I could have missed some)

Pg 52

line 5 delete 2nd period before Mutation

line 20 Allen, 2000 abbreviated Environ Mol Mutagen; Environmental *and* Molecular Mutagenesis

line 22 delete 2nd period before Mutation

line 35 Schilter not Schlter

line 45 abbreviated J Environ Sci Health C Environ Carcinog Ecotoxicol Rev

line 52 delete reference, identical to line 54 (Blakey not Blakely)

pg 53

line 17 Environmental *and* Molecular Mutagenesis

line 57 Environmental *and* Molecular Mutagenesis

pg 54

line 7 Environmental *and* Molecular Mutagenesis

line 29-31 delete, identical to previous

line 42 Williams not Wiiliams

line 45 Environmental *and* Molecular Mutagenesis

line 46 Williams not Wiiliams

line 49 Environmental *and* Molecular Mutagenesis
line 51 Environmental *and* Molecular Mutagenesis
line 54 Schuler not Schiuler

Pg 55

line 15 check if Blakely or Blakey
line 52 Jaekch R W?

Pg 56

line 1 Lefevre not Lefeure
line 26 Maurici not Muarici
line 27 Thybaud not Thybauld
line 49 Check Institute? concern;
line 50-51 abbreviated Food Chem Toxicol

Pg 57:

line 14 Environmental *and* Molecular Mutagenesis
line 13 Van Benthem J not Jorc?
line 21 *for 30 chemicals* not fir 30 chemiclas
line 26 Regulatory Toxicology *and* Pharmacology
line 31 Regulatory Toxicology *and* Pharmacology
line 33 abbreviated Chem Res Toxicol
line 42 2nd delete 2nd period before Mutation
line 55 Blakey not Blakely

Pg 58

line 1 Blakey not Blakely
line 18-20 OECD, include 487 (in vitro micronucleus)
line 25 Hayashi SU K (should be Hayashi M?)
line 33 Mailhes not Mailher

Pg 59

line 2-3 Environmental *and* Molecular Mutagenesis
line 4 Pacchierotti not Paccierotti
line 5 abbreviated Mut Res
line 9 Rothfuss not Roithfuss
line 10 abbreviated Chem Res Toxicol
line 22 abbreviated Chem Res Toxicol
line 24 Yauk not Yaulk

line 28 delete- identical to Singer 2006a
line 43 blood not blodd
line 47 delete- identical to Suzuki 2005a
line 53 delete second period before Mutagenesis

pg 60

line 4 abbreviated Mut Res
line 5 Blakey not Blakey
line 9 Blakey not Blakely
line 19 abbreviated Mut Res
line 27 Environmental *and* Molecular Mutagenesis
line 44 Environmental *and* Molecular Mutagenesis
line 53 delete 2nd period before Mutagenesis

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