COMMITTEE ON CARCINOGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

G07 - Alternatives to the 2-year Bioassay: An update

1. Guidance statement G07 – Alternatives to the 2-year Bioassay – discusses approaches that have been proposed as alternative strategies to the current paradigm using lifetime bioassays. G07 comprises four parts.

a. *in vivo* assays
b. cell transformation assays (CTAs)
c. developing methodologies (e.g. toxicogenomics)
d. alternative testing strategies incorporating results from short-term tests

2. Parts a and b have been published and are attached in Annex A, along with the general introduction to G07, for reference.

3. Two papers have been prepared for this meeting, to continue drafting of parts c. and d.

4. A short overview paper on toxicogenomics and high-throughput screening technologies (CC/2016/14) is presented as a basis for initial discussions relating to G07 part c.

5. A first draft of the Guideline Statement G07 part d is presented (CC/2016/15). This draft document is based on discussion paper CC/2016/07 that was considered at the COC meeting in July 2016.

Imperial College Toxicology Unit, supported by PHE
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COMMITTEE ON CARCINOGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT

G07 - Alternatives to the 2-year Bioassay: An update

This annex contains the current published version of the COC Guidance G07: Alternatives to the 2-year bioassay – part a) *In vivo* assays, and part b) Cell transformation assays.

The document is available here: https://www.gov.uk/government/collections/coc-guidance-statements

COC Secretariat
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Committee on CARCINOGENICITY

Committee on Carcinogenicity of Chemicals in Food, Consumer Products and the Environment (COC)

Statement COC/G07 - Version 1

Alternatives to the 2-year Bioassay

https://www.gov.uk/government/groups/committee-on-carcinogenicity-of-chemicals-in-food-consumer-products-and-the-environment-coc

COC Secretariat

c/o Public Health England

Centre for Radiation, Chemical and Environmental Hazards

Chilton, Didcot, Oxfordshire OX11 0RQ

Committee on CARCINOGENICITY

COC/G07 – Version 1

COMMITTEE ON CARCINOGENICITY OF CHEMICALS IN FOOD, CONSUMER PRODUCTS AND THE ENVIRONMENT (COC)

Alternatives to the 2-year Bioassay

General Introduction

1. This guidance statement comprises four parts, which together provide an overview of approaches that have been proposed as alternatives to the 2-year rodent bioassay for carcinogen risk assessment:

   a. *in vivo* assays
   b. cell transformation assays
   c. developing methodologies and strategies[1] (for example toxicogenomics)
   d. alternative testing paradigms[2] (for example, evaluation using histopathology and proliferative markers in sub-chronic rodent studies)

It is part of the Committee of Carcinogenicity (COC) guidance statement series which provides the Committee's views on all aspects of carcinogen risk assessment. It should be read in conjunction with G03 Hazard Identification and Characterisation: Conduct and Interpretation of Animal Carcinogenicity Studies.

2. The conduct of 2-year bioassays in two species, usually rat and mouse, has underpinned carcinogenicity risk assessment since the standard assay was developed in the 1960s (Cohen, 2010a,b). The objective of these long-term studies is to observe animals for the development of neoplastic lesions following exposure to a test substance for a major part of their life-span. The studies are usually designed to conform to closely defined test protocols and procedures (OECD GL 451 and 453, see Guidance Statement G03).

3. A significant body of data is available, particularly from the US National Toxicology Program (NTP), which has evaluated a large number of known carcinogens using the standard 2-year bioassay. Carcinogenicity testing strategies were developed taking into consideration the assumptions that, biologically, humans and animals are intrinsically similar and that carcinogenesis is a multistage process (Boobis et al., 2009). However, it has become evident that the conditions under which chemicals are tested are not necessarily relevant to human exposure, for example, the use of the maximum tolerated dose (MTD) or that some modes of carcinogenic action (MOA) are not relevant to human risk assessment. Furthermore,

[1] Part c) will be published at a later date
[2] Part d) will be published at a later date

standard carcinogenicity study protocols involve the use of large numbers of animals (approximately 400-500 of each species) and, with increasing concern surrounding unnecessary or poorly designed studies, efforts are being made to reduce animal use and to develop more refined testing strategies in line with the principles of 3Rs[3].

4. The use of both rat and mouse 2-year bioassays in assessing the carcinogenic potential of chemicals has been subjected to close scrutiny. Several detailed evaluations of datasets have been undertaken with a view to assessing the utility of the mouse bioassay and the relevance of non-genotoxic, liver only rodent carcinogens. In an assessment by Schach von Wittenau & Estes (1983) (cited by Alden et al., 1996), of 273 chemicals tested in both rats and mice, 206 were positive in both species whilst only 9 were positive in the mouse, and negative or inconclusive in the rat. Similarly, in an assessment by Huff et al (1991) (cited by Alden et al., 1996), 18 of 313 chemicals tested in both rats and mice in NTP studies were positive only in the mouse (i.e. 5.7%). Of 202 pesticides evaluated in the European Union, only 3 produced tumours only in mice (Billington et al., 2010). In a further review of data from 710 chemicals which had been tested in both the mouse and rat bioassays, only 3 compounds were identified as unequivocally non-genotoxic, mouse carcinogens in organs other than the liver. Mouse only, non-genotoxic liver carcinogenicity has been considered an unreliable indicator of human carcinogenic potential for some years (Osimitz et al., 2013).

5. These investigations and analyses suggest that a single two-year rodent assay is sufficient for cancer hazard identification. This view is endorsed by the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) which indicates that bioassay data from only one species (e.g. the rat) is required for evaluation of carcinogenic potential, when supported by appropriate mutagenicity and pharmacokinetic studies and a study from a short-term *in vivo* assay, such as a transgenic mouse model (ICH, 1998). As well as alternative *in vivo* models, *in vitro* cell transformation assays have been developed as alternative methods to detect carcinogenic potential, in particular for use in testing scenarios where *in vivo* testing is not permitted (e.g. cosmetics testing). Furthermore, recent developments in 'omics' technologies such as genomics, proteomics and metabolomics enable detailed examination of chemically-induced changes in the regulation of genes, proteins and metabolite profiles respectively. They are considered useful in providing insight into the mode and mechanism of action of the effects of chemicals, including carcinogenicity risk assessment.

6. The following parts of this Guidance Document present the Committee's opinions and views on the approaches with the potential to be used as alternatives to the 2-year rodent bioassay in a carcinogenicity testing strategy.

[3] https://www.nc3rs.org.uk/the-3rs

Alternatives to the 2-year bioassay

COC/G07: Part a) *In vivo* assays

A1. A number of alternative animal models have been developed for the prediction of carcinogenesis. In a regulatory setting, ICH Guideline S1B (ICH, 1998) supports the use of certain alternative models instead of a second species (usually, but not exclusively, the mouse) in the carcinogenicity testing strategy for the evaluation of human pharmaceuticals. It states the following:

> *"Several experimental methods are under investigation to assess their utility in carcinogenicity assessment. Generally, the methods should be based on mechanisms of carcinogenesis that are believed relevant to humans and applicable to human risk assessment. Such studies should supplement the long term carcinogenicity study and provide additional information that is not readily available from the long term assay. There should also be consideration given to animal numbers, welfare and the overall economy of the carcinogenic evaluation process. The following is a representative list of some approaches that may meet these criteria and is likely to be revised in the light of further information.*
>
> *a) The initiation-promotion model in rodents. One initiation-promotion model for the detection of hepatocarcinogens (and modifiers of hepatocarcinogenicity) employs an initiator, followed by several weeks of exposure to the test substance. Another multiorgan carcinogenesis model employs up to five initiators followed by several months of exposure to the test substance.*
>
> *b) Several transgenic mouse assays including the $p53^{+/-}$ deficient model, the Tg.AC model, the TgHras2 model, the XPA deficient model, etc.*
>
> *c) The neonatal in utero rodent tumorigenicity model"*

These three models are considered below for the current COC guidance in an order considered to be predominance of use.

i) Transgenic (Tg) animal models

A2. A number of genetically modified mouse strains have been developed with the aim of providing models to facilitate the quick and accurate detection of chemical carcinogens. The mice develop tumours much more rapidly than wild-type mice as the transgenic modifications involve genes critical to the carcinogenic process. This underpins their utility in risk assessment strategies. The Health and Environmental Sciences Institute (HESI) (part of The International Life Sciences Institute (ILSI)) co-ordinated a research and validation programme of work which evaluated the most commonly used models as part of the Alternative Cancer Test (ACT) programme: the $p53^{+/-}$ hemizygous knockout mouse, the rasH2 model, and the Tg:AC skin model.

The COC evaluated this programme of work and other alternative models for carcinogenicity testing (the Xpa$^{-/-}$ and Xpa$^{-/-}$ p53 $^{+/-}$ transgenic mouse models and the neonatal mouse model) in 2002 (COC, 2002)

A3. The overall conclusion was:
"*The COC agreed an overall conclusion that none of the models used in the ILSI/HESI Alternative Cancer Test programme were suitable as a replacement for the mouse carcinogenicity bioassay (the primary purpose for the development of these models) and that further research should look to identify models with a greater relevance to mechanisms of carcinogenicity in humans. Of the animal models assessed there was evidence that p53$^{+/-}$ transgenic mouse model could identify some genotoxic carcinogens. There was insufficient data to suggest that the other animal models under consideration (RasH2, Tg.AC, Xpa, Xpa/P53$^{+/-}$ and p53$^{+/-}$) provide essentially similar results."*

A4. Since the 2002 COC review, a number of studies and overviews evaluating the utility of the p53$^{+/-}$, RasH2 and Tg.AC models have been published and these have been considered for the current guidance. The Xpa/P53$^{+/-}$ model has not been considered as it is no longer commercially available.

p53$^{+/-}$ hemizygous knockout mouse

A5. p53$^{+/-}$ knockout mice are heterozygous for the tumour suppressor gene p53 - a point mutation in the remaining allele gives rise to a short latency period to tumour development. However, they have a low spontaneous tumour rate at 9 months thus making them sensitive to the detection of chemically-induced tumours, particularly those caused by genotoxic chemicals (French et al., 2001; Pritchard et al., 2003). The standard protocol involves daily oral dosing for 26 weeks, 5 dose groups including negative and positive controls, 25 mice/sex/group and extensive macroscopic and histopathological examination of tissues at the end of the study period. It is noted that it has been common practice to include a wild type control group at the high dose level and a wild type control group to establish whether any tumour response is related to inactivation of the p53 gene. The ILSI/ HESI project on ACT examined early assay performance, spontaneous tumour incidence and results of commonly used positive controls (e.g. p-cresidine) and provides a comprehensive evaluation of the assay (Storer et al., 2001). Of the 16 genotoxic human and/or rodent carcinogens evaluated, 12 were positive (75%). Seven putative non-human carcinogens, which are rodent carcinogens, were examined. Two were equivocal (chloroform, DEHP) and the other 5 were negative (chlorpromazine, haloperidol, metaproterenol, sulfamethoxazole, WY-14643). The three non-genotoxic, non-rodent carcinogens were negative.

A6. The p53$^{+/-}$ knockout model has demonstrated the ability to identify hormonal carcinogenic mechanisms (diethylstilboestrol (DES), 17β-oestradiol) and

immunosuppressive carcinogens (cyclosporine A), although the results are inconsistent (Storer et al., 2001; Alden et al., 2002). Some concerns have been raised within the pharmaceutical industry with regards to assay performance during a review of the use of the assay. This includes some negative results in the $p53^{+/-}$ model following a positive *in vitro* clastogenicity response (Storer et al., 2010). It is noted that the genetic background (i.e. mouse strain) can have an influence on the biological outcome, for example, TSG $p53^{+/-}$ mice treated with the laxative phenolphthalein developed lymphoma, whereas $p53^{+/-}$ mice from the CBA and CIEA strains did not (Okamura et al., 2003). More recently, an evaluation of 52 NTP-tested chemicals (37 positives, 15 negatives) showed that the concordance of $p53^{+/-}$ mouse with NTP mouse carcinogens was 57% (Eastmond et al., 2013). It was noted that there is no biological reason why the $p53^{+/-}$ model would not be able to detect non-genotoxic carcinogens. However, the conclusion that the model is sensitive to genotoxic carcinogens but not non-genotoxic carcinogens remains following further evaluations of the assay data (Jacobsen-Kram et al., 2004; Storer et al., 2010).

A7. The Committee notes that the $p53^{+/-}$ knockout model has been shown to give some unexpected results and is not considered to be reliable for detecting non-genotoxic carcinogens. US and EU regulatory authorities do not consider it to be an acceptable model to replace the 2-year rodent bioassay

rasH2 model

A8. The rasH2 model is a hemizygous transgenic mouse which carries the human *c-Ha-ras* gene with a point mutation and its own promoter elements (Morton et al., 2002). These mice develop spontaneous and chemically induced tumours more rapidly than their non-transgenic counterparts and this enhanced sensitivity to neoplasia underpins the rationale for the utility of this model for carcinogenic risk assessment. The standard protocol is essentially the same as for the $p53^{+/-}$ model with 25 mice/sex/group (Nambiar and Morton, 2013). A positive control response can be elicited by a single dose of N-methyl-N-nitrosourea (MNU).

A9. Data from the ILSI/HESI ACT trial indicate the utility of the rasH2 model for detecting both genotoxic and non-genotoxic chemicals (Usui et al., 2001). Two of the three genotoxic human carcinogens tested were positive (cyclophosphamide, phenacetin) whilst melphalan generated equivocal results. DES and 17β-oestradiol were positive and negative respectively, and the immunosuppressive cyclosporine A was equivocal. Of the 11 non-genotoxic rodent carcinogens tested, 10 were negative; clofibrate gave equivocal and positive results in two separate studies. Analyses of 37 IARC classified chemicals indicated an 81% accuracy when assessing assay performance with regards to human carcinogenicity (Pritchard et al., 2003). More recent test results provide some evidence that the rasH2 assay also has the capacity to identify some non-genotoxic rodent carcinogens (namely clofibrate, DEHP and WY-14643, ethylenethiourea, ethylacrylate, 1,4-dioxane,

troglitazone), though the majority of the assays of this class of chemicals were negative (Storer et al., 2010).

A10. A recent report reviews data from studies evaluating 10 chemicals in the rasH2 model in pharmaceutical laboratories and compared outcomes with the conclusions from 2-year rat bioassays (Nambiar and Morton, 2013). All chemicals tested were negative in genotoxicity tests. Two of the 10 chemicals were positive in the rasH2 model. Both of these chemicals were also positive in rat 2-year bioassays at the same histological sites and were also associated with proliferative findings in the target organs. Non-genotoxic MOAs were assumed for these chemicals. A review of the spontaneous tumours and histology in rasH2 mice from 11 studies indicated little variation in the background incidence and consistent qualitative and quantitative responses with MNU as the positive control (Nambiar et al., 2012). These studies provide control data, which aid the interpretation of studies and support the use of this model as an alternative to the mouse 2-year assay. Another review of NTP chemicals tested in mice indicated an overall 82% concordance of the rasH2 assay with the mouse 2-year bioassay (16/20 positives, 7/8 negatives) (Eastmond et al., 2013).

A11. The Committee concludes that the rasH2 model performs adequately and can be considered as an alternative to the mouse bioassay.

Tg.AC skin model

A12. Tg.AC transgenic mice are hemizygous for mutant *v-Ha-ras* and can be considered as genetically 'initiated' due to the presence of this transgene. This model differs from the other two models as the most commonly used protocol involves topical application of the test chemical and the induction of squamous cell papillomas or carcinomas as the endpoint (Tennant et al., 2001). The protocol comprises topical application of the test chemical 3 times per week for 26 weeks. Evaluation of the assay in the ILSI/HESI ACT indicated that the Tg.AC model detects both genotoxic and non-genotoxic human carcinogens but only 9/14 chemicals positive in a standard 2-year bioassay with a variety of carcinogenic modes of action demonstrated to be active in the model. The number of false positives was low (1/14), therefore the model was not considered over-sensitive (Tennant et al., 2001). However, more recent experience has shown that irritant chemicals, and some commonly used vehicle formulations which are slightly irritant, can increase the yield and latency of skin tumours (Lynch et al., 2007), therefore a positive result can be complicated by confounding inflammation.

A13. In a separate evaluation, 27/35 (77%) chemicals were accurately predicted for carcinogenesis (23 carcinogens, 12 non carcinogens) (Pritchard et al., 2003). A recent review indicates a 61% concordance between the Tg.AC assay and NTP mouse carcinogens (12/23 positives, 8/10 negatives) (Eastmond et al., 2013). It is considered that the Tg.AC model is able to predict both genotoxic and non-genotoxic

carcinogenesis when they are applied dermally. However, due to the persistent concerns with regards to tumourigenesis caused by inflammatory or irritant properties of chemicals the Tg.AC mice model is now generally considered unreliable for general use if interpretation of a positive result is complicated by confounding inflammation (Jacobs and Brown, 2015). Therefore its value in a carcinogenicity testing strategy is considered to be limited and accordingly it is not recommended.

Evaluation of the transgenic animal models

A14. A comprehensive overview and evaluation of all three assays ($p53^{+/-}$, Tg.AC and rasH2) used various combinations of the models, with and without consideration of rat 2-year bioassay data, to predict the carcinogenicity of 99 chemicals. It was concluded that correct identification of human carcinogens and non-carcinogens was 74-81% (whilst the similar evaluation of 2-year bioassay data was 69%). However some IARC Group 1 and Group 2A carcinogens were not identified and there were also a few false positives (Pritchard et al., 2003). A more recent evaluation of the three principal models suggests that, used alone, these assays would miss some probable human carcinogens (phenacetin, glycidol, 17β-oestradiol) (Storer et al., 2010). Furthermore, several issues of concern have been highlighted: methodological uncertainties, such as the effect of sample size on assay sensitivity and variability in spontaneous tumour frequencies, and reproducibility issues have been raised, together with questions on whether or how the dose-response data can be used for human risk assessment (Eastmond et al., 2013).

A15. A survey devised by the Carcinogenicity Alternative Mouse Models (CAMM) working group (Long et al., 2010) elicited 21 responses (90% of responses were from pharmaceutical organisations and 75% had used CAMM to support product development). The most commonly used model was the $p53^{+/-}$ mouse model with fewer laboratories using the rasH2 mouse model. There was only one example where the regulatory agency had rejected the submitted data. Feedback from agencies on study design was most often concerned with dose selection, in particular, whether the proposed high dose level was sufficiently close to the MTD to adequately ensure the sensitivity of the test. The most common positive controls used were p-cresidine for the $p53^{+/-}$ model, and urethane and MNU for the rasH2 model. However, it was considered by some respondents (5/15) that a positive control was not required if the model was well characterized within their laboratory. The tissues/organs which require pathological examination in the positive control animals are still under debate (i.e. all or only target organs). The importance of dose level selection was also discussed. Recommendations were proposed by the CAMM working group to improve study design and regulatory acceptance of transgenic animal studies.

Committee's evaluation of transgenic models

A16. The Committee's overall conclusion is that the rasH2 mouse model is the first choice model to replace the conventional mouse long-term bioassay as the assay has been shown to perform adequately for both genotoxic and non-genotoxic human carcinogens and is not overly sensitive. However, currently it is only supported when undertaken in addition to a rat 2-year bioassay (ICH, 1998). It is noted that, in a typical carcinogenic risk assessment strategy, chemicals with genotoxic properties will have been identified using the standard genotoxicity testing battery. Therefore the p53$^{+/-}$ assay is considered less useful than the rasH2 model as it is considered that it has an uncertain ability to predict chemicals with the potential to be carcinogenic in the absence of DNA reactivity. However, because of concerns about the insensitivity of C57BL p53$^{+/-}$ mice to detect non-genotoxic carcinogens, they have not been used routinely to test compounds which have given negative results in genotoxicity assays.

A17. The Committee notes that transgenic assays can also provide insight into carcinogenic mode of action. For example, they may be useful for investigating chemicals where high dose cytotoxicity or cell proliferation leads to the development of age-related tumours or where the carcinogenic MOA is attributable to pharmacodynamics action. Attention is drawn to the need for rigorous optimization of protocols and validation of study designs, and it is recommended that attempts are made to improve the understanding of false positives and negatives.

ii) In utero/neonatal exposure models of carcinogenesis

A18. The Committee evaluated the rat neonatal model of carcinogenesis in 1998 as part of the ICH initiative and the conclusions are provided in a statement (COC, 1999). It was noted that there were very limited validation data and the Committee concluded that the available information showed tumour yields with genotoxic carcinogens were highly dependent on the strain of animal, age at start of treatment, and treatment protocol. There were no validation data regarding the use of short-term neonatal rodent bioassays for the identification of non-genotoxic carcinogens. Overall, the Committee concluded that there was no current evidence to support the use of the neonatal mouse or rat bioassays as part of the regulatory testing strategy for human medicines. Data retrieved since the 1998 review were limited to mechanistic studies; for example, investigating arsenic-induced murine carcinogenesis (Tokar et al., 2011; Waalkes et al., 2006a; Waalkes et al.,2006b; Ahlborn et al., 2009).

Committee's evaluation of the in utero/neonatal model

A19. The Committee considers that whilst ICH Guideline S1B (ICH, 1998) allows the use of the neonatal mouse model, there are limited data available. The majority of the studies are investigations designed on a case-by-case basis and as such

there is no single protocol. The Committee concludes that the model is not relevant and not suitable as a general replacement for a 2-year bioassay.

iii) Initiation-Promotion models

A20. In the Solt Farber initiation-promotion model, rats are treated with a single dose of diethyl nitrosamine (DEN) as an initiator, followed by partial hepatectomy and repeated treatment with the test compound for several weeks to stimulate the formation of glutathione-S-transferase positive (GST+) foci which are considered to be pre-neoplastic lesions. The method was originally published in 1976 (Solt & Farber, 1976) and was developed and refined to become what is known as the Ito liver model (Ito et al., 1996; Ito et al., 2003). This is a medium-term treatment strategy and is based on the recognition that a large number of known carcinogens (genotoxic and non-genotoxic; >50% is quoted) are hepatocarcinogens in rodent bioassays and it is believed that the mode of action of many is mitogenic by stimulating hepatocyte proliferation. A multi-organ model based on the same principles was subsequently developed with the goal of identifying the carcinogens not detected by the Ito liver model.

A21. A single published study evaluating the model concluded that, of the 159 compounds tested, 61 of 66 rodent liver carcinogens were identified as positive, 10 of 43 compounds which were carcinogens but not in the liver (non-hepatocarcinogens) showed a positive result and 1 of 50 non-carcinogens was positive in this assay (Tsuda et al., 2010).

A22. Two published studies using the multi-organ initiation-promotion model described the testing of 44 chemicals. All of the 12 rodent liver carcinogens, 10 of the 11 non-hepatocarcinogens and 0 of the 1 non-carcinogens were positive in this assay (Fukushima et al., 1991; Ito et al., 1996).

A23. These models of carcinogenesis were developed over 40 years ago, and the Committee notes that there are few studies using this methodology published in the literature other than those published by the originators of the protocol.

Committee's evaluation of the initiation-promotion models

A24. The Committee concludes that initiation-promotion models are not suitable for use in a carcinogenicity testing strategy, but may be useful to investigate the mode of action of certain carcinogens.

COC
January 2016

Alternatives to the 2-year bioassay

COC/G07: Part b) Cell transformation assays

B1. The Committee on Mutagenicity (COM) recently undertook a detailed review of the available cell transformation assays (CTAs). The assays considered were: SHE pH6.7 or pH7.0; BALB/c 3T3; C3H10T1/2; and Bhas 43. A statement was produced in which it was concluded that, to date, the CTAs are not suitable for use in a regulatory testing strategy for carcinogenicity. However, they may have value in predicting rodent carcinogenicity if used in the scenario where *in vitro* positive results were obtained for a cosmetic ingredient and no *in vivo* testing is allowed. It is noted that the OECD is pursuing the improvement and validation of the cell transformation assays and the COM and COC are actively involved in monitoring and contributing to their development (COM, 2012).

B2. The COC accepts the conclusions reached by the COM.

COC
January 2016

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