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Clarification of some aspects related to genotoxicity assessment

EFSA Scientific Committee,
Anthony Hardy, Diane Benford, Thorhallur Halldorsson, Michael Jeger, Helle Katrine Knutsen, Simon More, Hanspeter Naegeli, Hubert Noteborn, Colin Ockleford, Antonia Ricci, Guido Rychen, Vittorio Silano, Roland Solecki, Dominique Turck, Maged Younes, Gabriele Aquilina, Riccardo Crebelli, Rainer Gürtler, Karen Ildico Hirsch-Ernst, Pasquale Mosesso, Elsa Nielsen, Jan van Benthem, Maria Carfì, Nikolaos Georgiadis, Daniela Maurici, Juan Parra Morte and Josef Schlatter

Abstract

The European Commission requested EFSA to provide advice on the following: (1) the suitability of the unscheduled DNA synthesis (UDS) *in vivo* assay to follow-up positive results in *in vitro* gene mutation tests; (2) the adequacy to demonstrate target tissue exposure in *in vivo* studies, particularly in the mammalian erythrocyte micronucleus test; (3) the use of data in a weight-of-evidence approach to conclude on the genotoxic potential of substances and the consequent setting of health-based guidance values. The Scientific Committee concluded that the first question should be addressed in both a retrospective and a prospective way: for future assessments, it is recommended no longer performing the UDS test. For re-assessments, if the outcome of the UDS is negative, the reliability and significance of results should be carefully evaluated in a weight-of-evidence approach, before deciding whether more sensitive tests such as transgenic assay or *in vivo* comet assay would be needed to complete the assessment. Regarding the second question, the Scientific Committee concluded that it should be addressed in lines of evidence of bone marrow exposure: toxicity to the bone marrow in itself provides sufficient evidence to allow concluding on the validity of a negative outcome of a study. All other lines of evidence of target tissue exposure should be assessed within a weight-of-evidence approach. Regarding the third question, the Scientific Committee concluded that any available data that may assist in reducing the uncertainty in the assessment of the genotoxic potential of a substance should be taken into consideration. If the overall evaluation leaves no concerns for genotoxicity, health-based guidance values may be established. However, if concerns for genotoxicity remain, establishing health-based guidance values is not considered appropriate.

Keywords: genotoxicity, *in vivo* UDS assay, micronucleus test, weight-of-evidence, bone marrow exposure

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Correspondence: sc.secretariat@efsa.europa.eu

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1. Introduction

Information on genotoxicity is a key component in hazard/risk assessment of chemicals in general, including those used in food and feed, consumer products, human and veterinary medicines, and industry. Genotoxicity testing of substances used or proposed for use in food and feed has been routine for many years. Genotoxicity information is also essential for risk assessment of natural and environmental contaminants in food and feed. Many regulatory agencies and advisory bodies have made recommendations on strategies for genotoxicity testing (EFSA Scientific Committee, 2011; ECHA, 2017). While the strategies for different chemical sectors may differ in points of detail, to evaluate genotoxic potential, the majority recommend use of a basic test battery comprising two or more *in vitro* tests, or *in vitro* tests plus an *in vivo* test. This is followed up when necessary, in cases where the results of basic testing indicate that a substance is genotoxic *in vitro*, by further studies to assess whether the genotoxic potential is expressed *in vivo*. Follow-up usually comprises one or more *in vivo* tests.

Optimisation of testing batteries to minimise false positives may reduce the likelihood of detecting inherent genotoxic activity. Thus, in recommending strategies for genotoxicity testing for risk assessment purposes, a balance needs to be struck that ensures with reasonable certainty that genotoxic substances likely to be active *in vivo* are detected.

1.1. Background and Terms of Reference as provided by the European Commission

Commission Regulation (EU) No 283/2013[1] sets out the data requirements for active substances for which dossiers were submitted on or after 1 January 2014 (i.e. applicable to all substances submitted under Regulation (EU) No 844/2012[2]) in accordance with Regulation (EC) No 1107/2009[3], and details the requirements in relation to genotoxicity testing in Section 5.4.

The requirements stipulate that the aim of genotoxicity testing is to

- predict genotoxic potential;
- identify genotoxic carcinogens at an early stage; and
- elucidate the mechanism of action of some carcinogens.

The requirements for *in vitro* studies are well defined, and certain studies must always be provided. Additionally, one *in vivo* study must always be provided even if all of the *in vitro* studies are negative, and furthermore, based on the results of the *in vitro* studies, further *in vivo* studies may also be required.

The data requirements stipulate that in cases where an equivocal or positive test result is obtained in any *in vitro* test, the nature of additional testing needed must be considered on a case-by-case basis, taking into account all relevant information covering the same endpoint as considered in the *in vitro* test.

The choice of *in vivo* studies is not prescriptively defined in the data requirements for all situations; rather, examples of possible suitable studies are provided. This is particularly the case for follow-up of positive *in vitro* gene mutation tests whereby the Transgenic Rodent Somatic (TGR) and Germ Cell Gene Mutation assays are listed as a possible follow-up.

Furthermore, the data requirements state that 'a tiered approach shall be adopted, with selection of higher tier tests being dependent upon interpretation of results at each stage'. Accepted tests and methods are listed in the Commission Communication,[4] which has been agreed by EFSA.

[1] Commission Regulation (EU) No 283/2013 of 1 March 2013 setting out the data requirements for active substances, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market. OJ L 93, 3.4.2013, p. 1.

[2] Commission Implementing Regulation (EU) No 844/2012 of 18 September 2012 setting out the provisions necessary for the implementation of the renewal procedure for active substances, as provided for in Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market. OJ L 252, 19.9.2012, p. 26.

[3] Regulation (EC) No 1107/2009 of the European Parliament and of the Council of 21 October 2009 concerning the placing of plant protection products on the market and repealing Council Directives 79/117/EEC and 91/414/EEC. OJ L 309, 24.11.2009, p. 1.

[4] Commission communication in the framework of the implementation of Commission Regulation (EU) No 283/2013 of 1 March 2013 setting out the data requirements for plant protection products, in accordance with Regulation (EC) No 1107/2009 of the European Parliament and of the Council concerning the placing of plant protection products on the market. OJ C 95, 3.4.2013, p. 1.

This therefore allows for some flexibility in terms of studies provided, depending on the characteristics of the substance. It should be further recalled that one of the general aims of EU law, also stated in Regulation 1107/2009, is to minimise the use of animal testing as far as possible. Testing on vertebrate animals should be replaced, restricted or refined, and only undertaken as a last resort.

The Scientific Opinion on genotoxicity testing strategies applicable to food and feed safety assessment[5] of the EFSA Scientific Committee (SC) provides recommendations for testing strategies for substances used in food and feed. The SC recommends a documented weight-of-evidence approach to the evaluation and interpretation of genotoxicity data, taking into account not only the quality and reliability of the data on genotoxicity itself, but also other relevant data that may be available. The Opinion also states that when considering pre-existing or non-standard data using a weight-of-evidence approach, a case-by-case approach using expert judgement is required.

During the evaluation of a number of active substances being considered under Regulation (EU) No 844/2012 (substances in the 'AIR3' renewal programme), a conclusion on the genotoxic potential of parent substances and/or metabolites (occurring in plants, livestock or groundwater) was not considered possible by EFSA based on the information submitted by the applicants in the dossiers considered in the peer review process. However, during the peer review process and thereafter, there have been strong divergences in opinion between some Member States, EFSA and applicants on this issue.

Taking into account the background above, the Commission requests consideration of the following three aspects to provide clarity and predictability for applicants and risk assessors carrying out assessments of genotoxicity and also for risk managers:

1) The adequacy of the Unscheduled DNA Synthesis (UDS) Assay to follow-up positive results in the *in vitro* gene mutation tests;

In light of this, the following questions are raised:

   a) Whilst it is clear that the Opinion of the SC does not recommend the UDS Assay as an appropriate follow-up study, in what situations and for what types of substances is the UDS Assay (carried out prior to the Opinion of the SC and submitted in a dossier for approval/ renewal of approval) suitable to follow-up a positive *in vitro* gene mutation result?
   b) Does the current Opinion of the SC on testing strategies[5] satisfactorily highlight the limitations of using the UDS Assay in the assessment of genotoxicity? Does it fully consider how the UDS Assay may be used (when submitted as part of a data package that was developed prior to the Opinion)?
   c) Can any further indicators or advice be provided to better enable risk assessors to consider if a submitted UDS Assay is adequate to be used in concluding on the genotoxic potential of a substance?

2) The adequacy to demonstrate target tissue exposure in *in vivo* studies, particularly in the micronucleus (MN) test

   a) What type of measurements or information from other studies can be used to provide reassurance of bone marrow exposure in MN or other studies and enable the use of such studies to conclude on the genotoxic potential of the substance e.g. clinical signs of toxicity (particularly those considered systemic in nature), presence in the blood/plasma, ADME data?
   b) In the absence of information to definitively confirm bone marrow exposure, what additional data or information can be used to allow (if possible) for the use of such studies in the assessment of genotoxicity and provide reassurance that a negative result is not a false negative?

3) The use of data in a weight-of-evidence approach to conclude on the genotoxic potential of substances and the consequent setting of health-based reference values for use in human health risk assessment.

The EFSA Scientific Committee recommends in its 2011 Opinion:

> 'a documented weight-of-evidence approach to the evaluation and interpretation of genotoxicity data, in particular when considering heterogeneous and non-standard data sets

[5] EFSA Journal 2011;9(9):2379, 69 pp.

[particularly relevant for considering contaminants]. 'Such an approach should not only consider the quality and reliability of the data on genotoxicity itself, but also take into account other relevant data that may be available, such as physico-chemical characteristics, structure-activity relationships (including structural alerts for genotoxicity and read-across from structurally related substances), ADME, and the outcomes of any repeat-dose toxicity and carcinogenicity studies'.

a) It should be further considered and clarified as to what this means in terms of performing robust assessments of genotoxicity for pesticide active substances and metabolites in the absence of the standard or preferred battery of tests (and/or when the recommendations in the Opinion on genotoxicity testing are not followed), or where there may be positive results *in vitro* but where negative results in *in vivo* studies may have some limitations. In particular, this should be specifically considered for cases where there is a complete and robust data set available, including carcinogenicity and reproductive toxicity studies. This point should be considered in light of the need to minimise animal testing.

Taking this into account, the following point should be considered:

b) What considerations should be taken into account as part of a weight-of-evidence approach when determining if toxicological reference values for use in health-based risk assessments can be set when there are uncertainties or inadequacies with the assessment of genotoxicity (non-standard/non-preferred data set or non-guideline studies) and in which circumstances, if any, is it considered not possible to set health-based reference values, either fixed or provisional?

The Commission requests consideration of these three questions to provide clarity and predictability for applicants and risk assessors carrying out assessments of genotoxicity and also for risk managers when making decisions on approval of substances. The genotoxicity data requirements in the different EFSA frameworks, the Opinion of the SC on genotoxicity testing strategies and the wider scientific and technical knowledge on the assessment of genotoxicity relevant to all compounds potentially present in food in the different EFSA frameworks shall be also taken into account.

1.2. Interpretation of the Terms of Reference

The mandate of the European Commission was focusing mainly on active substances of plant protection products, however, the SC considers that this opinion is relevant also to other areas within EFSA's remit.

Although, there is a clear notion that there is no need for *in vivo* testing when all *in vitro* genotoxicity tests (as specified in the EFSA SC opinion of 2011) are negative, the SC noted that there is always a legal requirement for *in vivo* test(s) for active substances in plant protection products.

When addressing question 2 of the mandate, the SC assumes that the *in vivo* MN test has been selected as the appropriate follow-up of a positive *in vitro* outcome (aneugenicity/clastogenicity). This opinion will not address follow-up testing strategies of a positive *in vitro* test outcome. Hence, the interpretation of the question of the mandate is how to verify the exposure of the bone marrow when performing an *in vivo* MN test, either measuring micronuclei in bone marrow cells or in peripheral blood cells, and which elements (lines of evidence) should be taken into consideration to provide reassurance that a negative result in the *in vivo* MN test is not a false negative. The SC decided to categorise the elements in lines of evidence of exposure of the bone marrow.

When addressing question 3 of the mandate, 'setting of health-based reference values for use in human health risk assessment' it is understood as establishing health-based guidance values (HBGV).

The SC notes that the quantitative assessment of dose-response relationships in genotoxicity studies is beyond the scope of the present Opinion.

1.3. Additional information

According to the genotoxicity testing strategies proposed by EFSA (EFSA Scientific Committee, 2011), when all *in vitro* endpoints are clearly negative, it can be concluded that the substance is not genotoxic. If *in vivo* testing is considered necessary to follow-up a positive *in vitro* outcome, the choice of *in vivo* tests should relate to the genotoxicity endpoint(s) identified by the aforementioned positive *in vitro* tests as well as to the appropriate target tissues.

The SC notes that for different areas of risk assessments conducted by EFSA, the basis for evaluation of genotoxicity may differ with respect to the extent of the available dataset and its robustness. In the context of this Opinion, three different situations are considered:

1) Regulated 'new' substances that are being subject to risk assessment in the context of a (first) authorisation procedure and for which a standard data package is required per regulation, e.g. new pesticide active substances and new food additives. For such substances, an extensive data set, including studies that have been conducted according to the most recent standard of testing guidelines, can be expected.
2) Regulated 'old' substances that have undergone assessment and approval in the past and are currently under re-evaluation with respect to extension of authorisation or renewal of approval, e.g. pesticide active substances under the renewal procedure and renewal of food/feed additives. Although a defined extensive data package is also required for such substances, the submitted dossiers may contain studies that were conducted under older protocols differing from updated protocols, or under provisions not reflecting the current state of scientific development or current data requirements.
3) Substances for which the available dataset is not based on data requirements laid down in specific legislation and thus may often contain information gaps and/or information from non-standard (non-guideline) studies (e.g. in the case of contaminants or metabolites in plants).

Taking into account the emphasis within the scope of the Terms of Reference, the current Opinion will focus mainly on situations II and III.

2. Data and methodologies

2.1. Data

The evidence used for this mandate stems primarily from expert knowledge gathered by a working group of the EFSA SC dedicated to the work of this Opinion. Recent ongoing activities at different national and international levels were considered and discussed. The analyses presented in the appendix of this Opinion rely on the data from the EURL ECVAM database (https://eurl-ecvam.jrc.ec.europa.eu/databases/genotoxicity-carcinogenicity-db) as well as data retrieved from the literature. The EURL ECVAM database includes 726 Ames positive substances representing 747 unique CAS numbers when salts and isomers were combined. The data originated from several sources (e.g. EFSA, ECHA, NTP). The database includes 141 substances with UDS data, of which 78 are carcinogens. Among these 78 chemicals, 24 (30%) overlap with the Kirkland and Speit, 2008 database. In relation to the analysis of the 19 carcinogens in the ECVAM database that were commonly tested in the UDS, transgenic rodent (TGR) and/or comet assays, 9 overlapped with the Kirkland and Speit, 2008 database.

Established genotoxicity testing strategies are described in 'Scientific Opinion on Genotoxicity testing strategies applicable to food and feed safety assessment' (EFSA Scientific Committee, 2011). Following the EFSA policy on transparency and engagement in risk assessment (TERA), EFSA decided to publish the draft Opinion for public consultation to get additional data and views from interested stakeholders. The draft was then revised and finalised, to be tabled for adoption at the 86th SC plenary meeting in November 2017.

2.2. Methodologies

The methodology used for this Opinion was to aggregate the information from the different EFSA areas where the assessment of genotoxicity is performed, considering different legislation applicable in the food and feed area, relevant EFSA scientific opinions and guidance documents and information from the open literature. Ongoing activities in other EU or international organisations were considered. The working group of the SC was composed of members of the SC and members of the panels dealing with genotoxicity assessment. *Ad hoc* hearing experts were invited to provide additional data specifically in relation to questions 1 and 2 of the mandate. Consultation with the European Chemicals Agency (ECHA) took place prior to the endorsement by the SC of the draft Opinion for public consultation and during the finalisation of the opinion. The public consultation was launched on the EFSA website on 24 July 2017 and ended on 6 September 2017. The report of the public consultation summarising the comments received and a summary on how they have been addressed will be published by the end of 2017.

3. Assessment

3.1. The adequacy of the unscheduled DNA synthesis (UDS) assay to follow-up positive results in the *in vitro* gene mutation tests

The rat liver unscheduled DNA synthesis assay, referred hereafter as UDS, detects the induction of DNA repair synthesis in the liver of treated adult rats. To this aim, the incorporation *ex vivo* of radioactive-labelled nucleotide, e.g. tritium-labelled thymidine (^{3}H-TdR), is measured in isolated hepatocytes by autoradiography. Because the rate of liver cell proliferation is very low in adult animals and the few replicating cells can be easily identified, the incorporation of radioactivity in nuclei of non-replicating cells indicates unscheduled DNA synthesis related to the repair of DNA by excision, removal and replacement of a damaged stretch of a DNA strand. The UDS is thus an indicator test detecting DNA damage, not a mutagenicity assay measuring stable genetic alterations. The test is designed to respond only to substances that induce a type of DNA damage that is repaired by excision repair; DNA damage processed by other mechanisms, as well as unrepaired genetic damage are not detected with this assay.

In principle, a UDS could be applied to any tissue not undergoing extensive cell proliferation, but in practice most experience is related to its application in the rat liver. A protocol for the detection of UDS in rat liver cells was first proposed by Mirsalis et al. in the 1980s (Mirsalis and Butterworth, 1980; Mirsalis et al., 1982). Based on the original study protocol (Butterworth et al., 1987), an OECD Test Guideline (TG 486: Unscheduled DNA synthesis (UDS) test with mammalian liver cells *in vivo*) was developed and adopted in 1997, and never updated thereafter (OECD, 2017). The TG 486 has been confirmed but not updated among the OECD Genetic Toxicology Test Guidelines following their recent update (2014–2015), even though it was noted that 'The test responds positively only to chemicals that induce the type of DNA damage that is repaired by nucleotide excision (mainly bulky adducts)... (OECD, 2016a–f)'.

Based on the DNA lesion and tissue specificity, it is widely accepted that negative results in the UDS is insufficient alone to rule out an *in vivo* genotoxic potential. Nevertheless, the UDS was widely applied in studies performed for regulatory purpose until the adoption of the OECD test guidelines for the transgenic rodent somatic and germ cell gene mutation assays (TGR, OECD TG 488, 2013) and the *in vivo* mammalian alkaline comet assay (OECD TG 489, 2014). Both TGs have been updated in 2013 and 2016,[6] respectively. As a matter of fact, until then the UDS was the only test method applicable to somatic tissues other than the erythropoietic system, addressed by cytogenetic tests (OECD TGs 474 and 475). Thus, even though in principle the UDS should be considered appropriate only for substances targeting the liver and/or requiring liver metabolic activation, and inducing adducts removed by excision repair, it was formerly frequently recommended in the follow-up of substances positive in gene mutation tests *in vitro* (e.g. Ames test, mammalian gene mutation tests – *hprt* or *TK* genes (OECD, 2015)). According to early genotoxicity testing strategies, negative results in at least two *in vivo* assays in different target tissues were required in the follow-up of *in vitro* positives (Carere et al., 1995; online) (see also Appendix A), and the UDS was often performed by default as complement of cytogenetic assays in erythropoietic cells.

Nowadays the TGR and *in vivo* comet assays play a prominent role in genotoxicity testing strategies compared to the UDS. According to the ECHA guidance on information requirements for safety assessment (2017), 'The TGR and comet assays offer greater flexibility than the UDS test, most notably with regard to the possibility of selecting a range of tissues for study on the basis of what is known of the toxicokinetics and toxicodynamics of the substance'. Therefore, ICH and ECHA propose that the application of the UDS for the follow-up of *in vitro* positives should be justified case-by-case, based on knowledge of target organ specificity, metabolism, and DNA adduct formation (ICH, 2014; ECHA, 2017) In particular, ECHA guidance states that 'the UDS should be used only when it can reasonably be assumed that the liver is the target organ, since the UDS is restricted to the detection of primary DNA repair in liver cells'.

Also according to the EFSA SC Opinion on genotoxicity testing strategies '...UDS has a limited use for cells other than liver, and its sensitivity has been questioned'. (EFSA Scientific Committee, 2011). Accordingly, the UDS assay was not included among recommended test methods in more recent EFSA guidance document, on food additives (EFSA ANS Panel, 2012) and food contact materials (EFSA CEF Panel, 2008).

[6] In the case of the comet assay, the reason for the update was only to include the reference to the document presenting the set of OECD TGs on genotoxicity which was approved in April 2016.

The analysis of genotoxicity databases supports the above conclusions on the limited regulatory use of the UDS. A first critical assessment of the role of the UDS in regulatory testing was performed by Kirkland and Speit (2008). In their analysis, within a set of rodent carcinogens negative or equivocal in the bone marrow MN test, less than 20% (7 out of 41) tested positive in the UDS. Conversely, the respective figures for TGR and comet assay are more than 50% and about 90% (Kirkland and Speit, 2008). Compounds testing negative in the UDS included 19 rodent liver carcinogens, 6 of which were positive in the *in vivo* comet assay. The authors noted that even narrowing the analysis to carcinogens inducing gene mutations *in vitro* – to which the UDS is expected to be more responsive – a high incidence of negative responses was observed.

A further analysis was performed to support this Opinion using data from the EURL ECVAM database (EURL EVCAM, 2017). This recently constructed database, which includes part of the Kirkland and Speit data set, compiles available genotoxicity and carcinogenicity data for 726 Ames-positive chemicals covering different sectors (industrial chemicals, cosmetics ingredients, plant protection and pharmaceutical products) and originating from different sources (regulatory agencies, industry and publicly available literature).[7] A rigorous methodology and defined criteria were applied for the selection and analysis of the data (Kirkland et al., 2014). Only chemicals with a known chemical identity and validated *in vitro* and *in vivo* results for the genotoxicity endpoints and/or carcinogenicity were selected. Data for the following tests were collected: *in vitro* tests (Ames, gene mutation using TK gene or gene mutation using Hprt/xprt genes, MN, chromosome aberration (CA)); *in vivo* tests (MN, CA, UDS, TGR, DNA breakage (comet and alkaline elution assay)); rodent carcinogenicity. 'Overall Calls' (Appendix A) were defined for each genotoxicity assay *in vitro* and *in vivo,* and carcinogenicity by following defined criteria for the reliability of each study and quality of data for those chemicals appearing in more than one source with different calls. Four categories were considered: positive, negative, equivocal and inconclusive. Where information was missing, even for those chemicals with one single data entry, scientific literature was consulted. The data were analysed by calculating the sensitivity and specificity of the respective tests.

The database comprises 87 Ames-positive chemicals that were tested both in the UDS *in vivo* and for carcinogenicity. Ten of these chemicals were not carcinogenic. Of the 77 Ames-positive that are carcinogens and tested for UDS, 46 were positive in UDS. Thus, the sensitivity of the UDS to predict (genotoxic) carcinogenicity using this data set was 59.7% (Appendix B, Table B.1). By contrast, when performing a similar analysis, the sensitivity of TGR and the comet assay to predict carcinogenicity was 88.2% (67 positives out of 76 carcinogens) (Appendix B, Table B.2) and 91.2% (52 positives out of 57 carcinogens), respectively (Appendix B, Table B.3). However, when the analysis was restricted to a common set of carcinogens, no distinct difference in sensitivity between UDS and TGR assay was observed (Appendix B, Table B.4), However, the SC noted that this subset of intensively tested compounds mainly consists of powerful genotoxic carcinogens (Appendix B, Table B.5), which may provide a biased estimate of the true sensitivity to carcinogens of individual genotoxicity assays.

The EURL ECVAM database also allows assessment of the concordance of results from different *in vivo* genotoxicity assays. This is considered a relevant contribution to the comparative assessment of the performance of the assays, as the *in vivo* genotoxicity represents a relevant toxicological end-point *per se*, independent of the outcome of carcinogenicity tests (EFSA Scientific Committee, 2011).

In this respect, the EURL ECVAM database comprises 111 Ames-positive substances tested both in the UDS and in at least one other *in vivo* genotoxicity test (MN, CA, TGR, comet). Sixty-six substances that were Ames-positive and *in vivo* positive (in either MN, CA, TGR or comet assay) were also tested in the UDS: out of these, 39.4% (26/66) were negative in the UDS; conversely, among 45 Ames-positive substances that were negative *in vivo* (in either MN, CA, TGR or comet assay), only 4 (8.8%) were positive in the UDS. Overall, this analysis shows an average concordance of results between UDS and other *in vivo* assays of 73% (81/111), with a remarkable number of *in vivo* positives 26/66 (39.4%) not detected by the UDS (Appendix B, Table B.6).

Overall, the low sensitivity of the UDS in detecting rodent carcinogens and/or *in vivo* genotoxicants highlighted by the analysis of the EURL ECVAM database confirms previous conclusions on the lower predictive value of the UDS compared with TGR and *in vivo* comet assays provided by the analysis of a smaller data set (Kirkland and Speit, 2008), supporting a more prominent role for the latter assays in regulatory testing strategies, as already recommended in most guidance documents (e.g. EFSA Scientific Committee, 2011; ECHA, 2017).

[7] It needs to be noted, however, that the database is biased towards positive carcinogenic and genotoxic chemicals.

With reference to the specific question on the suitability of the UDS addressed in the Terms of Reference, the SC concluded that, taking into consideration the current EU legislation and guidance documents, this question should be addressed in both a retrospective and a prospective way.

- For future assessments (new submissions), the EFSA SC is not aware of situations or substances where the UDS could be considered preferable to the TGR or comet assay (point (a) of the Terms of Reference). Therefore the use of the UDS is no longer recommended as a follow up of positive *in vitro* tests.
- For re-assessment, in cases where UDS data as a follow-up to a positive *in vitro* mutation test already exists, there might be positive or negative results: test results may be considered as adequate to assess genotoxic potential only in cases of positive results. If the outcome of the UDS is negative, the reliability and significance of results should be carefully evaluated in a weight-of-evidence (WoE) approach, taking into account all available information on mode of action (e.g. the type of DNA damage), metabolism, toxicokinetics, etc., before deciding whether more sensitive tests such as TGR or *in vivo* comet assay would be needed to complete the assessment (see also Section 3.3).

3.2. The adequacy to demonstrate target tissue exposure in *in vivo* studies, particularly in the mammalian erythrocyte Micronucleus test

As mentioned in Section 1.2, the SC assumes that the *in vivo* mammalian erythrocyte micronucleus (MN) test (OECD TG 474) has been selected as the appropriate test to follow-up a positive *in vitro* outcome. The SC notes that in this context appropriate means *in vivo* testing for the same endpoint as observed *in vitro.*

If a positive result is observed in the *in vivo* mammalian erythrocyte MN test, demonstration of target tissue exposure is not needed. However, evidence of bone marrow exposure is needed to conclude that a substance is not genotoxic based on a negative mammalian erythrocyte MN test outcome.

Regarding bone marrow exposure, the OECD Test Guideline (TG) 474 (July 2016), states:

- *'A blood sample should be taken at appropriate time(s) in order to permit investigation of the plasma level of the test substances for the purposes of demonstrating that exposure of the bone marrow occurred, where warranted and where other exposure data do not exist';*
- *'[…]. Evidence of exposure of the bone marrow to a test substance may include a depression of the immature erythrocyte ratio or measurement of the plasma or blood level of the substance. In case of intravenous administration, evidence of exposure is not needed. Alternatively, ADME data, obtained in an independent study using the same route and same species, can be used to demonstrate bone marrow exposure. […]'*

If there is evidence from the mammalian erythrocyte MN test that the test substance induces toxic effects in the bone marrow, it can be concluded that the substance has reached the bone marrow.

Moreover, as the bone marrow is a well-perfused tissue, systemic bioavailability of a test substance can be considered as a line of evidence[8] of bone marrow exposure. Evidence on systemic bioavailability can also be obtained from toxicity studies when test-substance-related systemic toxicity is observed. Therefore, the following lines of evidence could be considered.

- toxicity to the bone marrow observed in the mammalian erythrocyte micronucleus test;
- toxicity to the bone marrow observed in toxicity studies;
- test substance (and/or metabolites) detected in the bone marrow in a toxicokinetic study;
- systemic toxicity observed in the bone marrow micronucleus test;
- systemic toxicity observed in toxicity studies;
- test substance (and/or metabolites) detected systemically in a toxicokinetic study;
- test substance detected systemically in a specific blood/plasma analysis.

These different lines of evidence of bone marrow exposure are described shortly below.

[8] A **line of evidence** is a set of evidence of similar type (EFSA Scientific Committee, 2017a).

3.2.1. Lines of evidence of bone marrow exposure

3.2.1.1. Toxicity to the bone marrow observed in the mammalian erythrocyte Micronucleus test

The main line of evidence is toxicity to the bone marrow indicated as a reduction in the proportion of immature erythrocytes among total erythrocytes, i.e. a decrease in the PCE/(NCE+PCE) ratio (PCE: polychromatic erythrocytes, i.e. immature erythrocytes, also named reticulocytes; NCE: normochromatic erythrocytes, i.e. mature erythrocytes). A decrease in the ratio in such a study, if evaluated as being test-substance related, is sufficient evidence of bone marrow exposure.

3.2.1.2. Toxicity to the bone marrow observed in toxicity studies

In repeated-dose toxicity studies performed according to an internationally accepted test guideline such as the oral 28-day or 90-day studies (OECD TG 407 (OECD, 2008), TG 408 (OECD, 1998a), TG 409 (OECD, 1998b)) and the chronic toxicity studies (OECD TG 451 (OECD, 2009a), TG 452 (OECD, 2009b), TG 453 (OECD, 2009c)), the bone marrow is one of the tissues that should be examined histopathologically. A histopathological change in the bone marrow observed in such a study using the same route and the same species as in the mammalian erythrocyte MN test, if evaluated as being test-substance related, is a line of evidence of bone marrow exposure. If the toxicity data used refers to a different species, a rationale (substantiated by data) for considering this data representative of the species used in the genotoxicity study must be presented.

3.2.1.3. Test substance detected in the bone marrow in toxicokinetic studies

Toxicokinetic studies (OECD TG 417 (OECD, 2010)) are conducted to obtain information on absorption, distribution, biotransformation (i.e. metabolism) and excretion of a test substance. Generally, in toxicokinetic studies, the test substance is administered as the ^{14}C-radiolabelled compound (with the radiolabel located in a core portion of the molecule that is metabolically stable) and the radioactivity measured in the blood/plasma, organs and tissues, and excreta. Detection of radioactivity in the bone marrow in such a study, using the same route and the same species, provides a line of evidence of bone marrow exposure to the test substance itself and/or its metabolites. If the toxicokinetic data used refers to a different species, a rationale (substantiated by data) for considering this data representative of the species used in the genotoxicity study must be presented.

3.2.1.4. Systemic toxicity observed in the bone marrow micronucleus test

In the bone marrow MN test (OECD TG 474), general clinical observations of the test animals (cage-side) should be made and clinical signs recorded at least once a day. Certain clinical signs, which will usually only occur if the test substance is systemically bioavailable (such as certain signs related to the central nervous system (CNS), e.g. change of spontaneous activity, ataxia, sedation, staggering), if evaluated as being test-substance related, is one line of evidence of systemic bioavailability and indicates bone marrow exposure.

3.2.1.5. Systemic toxicity observed in toxicity studies

Systemic effects observed in repeated-dose toxicity studies, using the same route and the same species as in the mammalian erythrocyte MN test, if evaluated as being related to the test-substance or to its metabolites, are lines of evidence of systemic bioavailability, and can be considered as a line of evidence of bone marrow exposure. Some examples of endpoints which might be lines of evidence are listed in Appendix C.

3.2.1.6. Test substance detected systemically in toxicokinetic studies

By analogy to what is described in Section 3.2.1.3, detection of radioactivity in the blood/plasma, organs and tissues, or urine in an ADME study using the same route and the same species, provides sufficient evidence of systemic bioavailability of the test substance itself and/or its metabolites, and consequently, is a line of evidence of bone marrow exposure to the test substance itself and/or to its metabolites.

3.2.1.7. Test substance detected systemically in a specific blood/plasma analysis

According to the revised version of the mammalian erythrocyte MN TG (OECD TG 474) adopted in July 2016 'A blood sample should be taken at appropriate time(s) in order to permit investigation of

the blood/plasma level of the test substances for the purposes of demonstrating that exposure of the bone marrow occurred, where warranted and where other exposure data do not exist'. Detection of the test substance in a specific blood/plasma analysis above the quantification limit is sufficient evidence of systemic bioavailability of the test substance and, therefore, could be considered as a line of evidence of bone marrow exposure.

The following aspects to evaluate whether the specific blood/plasma analysis is suitable to indicate systemic exposure should be considered before concluding on the validity of the analysis:

- Are the blood/plasma samples taken at the appropriate times, i.e. is the documentation for blood/plasma sampling times justified in terms of the toxicokinetics of the test substance – e.g. rapid elimination of the test substance and/or its metabolites from blood/plasma and distribution to organs and tissues, rapid metabolism of the test substance – if only the parent compound is measured?
- Are the test substance itself and/or its metabolites measured?
- Can the analytical method only detect the free test substance or can it account for both the free and the bound test substance if a test substance is reversibly or irreversibly bound to e.g. blood/plasma proteins?
- Is the analytical method appropriate, i.e. can it detect and quantify the actual substances to be measured for the particular evaluation (are the limit of detection, the limit of quantification and the calibrated range reported for the analytical method used and are these parameters considered adequate)?
- Is extrapolation between species necessary, e.g. is the specific plasma analysis performed in a rat study whereas the actual mammalian erythrocyte MN test is performed in mice?
- Are the blood/plasma levels measured within or below the calibrated range?
- Are the blood/plasma levels plausible considering, e.g. consistency among animals of the same group (i.e. how large is the variation) and among different sampling times?

3.2.2. Assessment of the different lines of evidence regarding bone marrow exposure

The SC notes that toxicity to the bone marrow (as detailed under Sections 3.2.1.1 or 3.2.1.2) in itself may provide sufficient evidence of bone marrow exposure to conclude on the validity of a negative outcome of a study based on the *in vivo* mammalian erythrocyte MN assay.

All other lines of evidence of bone marrow exposure should be assessed within a WoE (EFSA Scientific Committee, 2017a) to decide whether they might provide sufficient reassurance of a valid negative test result, taking dose and timing into account. In this context, it should be assessed on a case-by-case basis whether the detection of any (radioactive) test substance in bone marrow or the detected levels in blood/plasma might be biologically relevant to the target tissue (EFSA Scientific Committee, 2017b).

3.2.3. Additional information for the assessment of genotoxicity following a negative result in the mammalian erythrocyte micronucleus test, in the absence of confirmation of bone marrow exposure

In the absence of a confirmation of bone marrow exposure, further testing should be performed, according to the testing strategy outlined in the EFSA Scientific Committee, 2011 opinion.

The SC is aware of international discussions about non-validated *in vivo* MN tests in tissues other than bone marrow and peripheral blood, such as in the liver and in tissues of the gastro-intestinal tract (e.g. stomach or colon). Although they could provide useful scientific information for substances that are positive in *in vitro* MN tests, further actions for development are needed to fully establish the methods and identify their sensitivity and specificity (Report of the 6th International Workshop on Genotoxicity Test Procedures; Martus et al., 2015; Uno et al., 2015a,b).

3.3. The use of data in a weight-of-evidence approach to conclude on the genotoxic potential of substances and the consequent setting of health-based guidance values for use in human health risk assessment

3.3.1. General considerations on weight-of-evidence

The WoE approach is defined by EFSA Scientific Committee (2017a) as a process in which evidence is integrated to determine the relative support for possible answers to conclude on the validity or informative value of individual studies or in assessing specific endpoints such as genotoxicity. It comprises three basic steps: (1) assembling the evidence into lines of evidence of similar type, (2) weighing the evidence, (3) integrating the evidence, also at a higher level, i.e. integrating all endpoints within the overall hazard assessment. Furthermore, all EFSA scientific assessments must include consideration of uncertainties, reporting clearly and unambiguously what sources of uncertainty have been identified and what their impact on the assessment outcome is.

In line with both the Scientific Opinion on genotoxicity testing strategies and Guidance on Weight of Evidence (EFSA Scientific Committee, 2011, 2017a), the SC recommends 'a documented weight-of-evidence approach for the evaluation and interpretation of genotoxicity data', taking into account not only the quality and availability of the data on genotoxicity itself, but also all other relevant data that may be available. The SC notes that for different areas of regulatory risk assessment, the basis for evaluation of genotoxicity may differ with respect to the extent of the available dataset and its robustness. When considering pre-existing or non-standard data, a case-by-case approach using expert judgement is required which should include justifying the choice of methods used, documenting all steps of the procedure in sufficient detail and making clear where and how expert judgement has been used. Reporting should also include referencing and, if appropriate, listing or summarising all evidence considered, identifying any evidence that was excluded; detailed reporting of the conclusions; and sufficient information for readers to understand how the conclusions were reached. The WoE assessment and uncertainty are closely related. The expression of uncertainty for the conclusion of the assessment of genotoxicity as a whole should include any uncertainties associated with the WoE process itself.

The SC notes that lacking or inadequate genotoxicity data cannot be substituted with data from studies involving other endpoints including carcinogenicity and reproductive toxicity, as genotoxicity is an endpoint *per se*. Besides providing information about potential irreversible DNA damage that may result in heritable or degenerative diseases, genotoxicity data are also used to predict potential carcinogenicity (EFSA Scientific Committee, 2011). On the other hand, data demonstrating carcinogenicity may contribute to the genotoxicity WoE.

3.3.2. Drawing conclusions on the genotoxic potential of substances

Evaluation of all available *in vitro* and *in vivo* results of the standard test battery according to the published EFSA SC Opinion on genotoxicity testing strategies (EFSA Scientific Committee, 2011) and according to the EFSA SC Guidance on the use of the WoE approach in scientific assessments (EFSA Scientific Committee, 2017a) is the first recommended step to conclude on the genotoxicity of substances. This is applicable to the re-evaluations as well as to the first evaluation of a substance. Particular studies belonging to the standard or preferred battery of genotoxicity tests (EFSA Scientific Committee, 2011) may not have been conducted according to the revised OECD guidelines from 2016 or may not be available (e.g. TGR and comet assay). If the data set includes any of the two assays specifically addressed in questions 1 and 2 of the Terms of Reference (liver UDS assay *in vivo*, mammalian erythrocyte MN test *in vivo*), the advice provided in answering those questions should be taken into consideration for the assessment of the informative value of the resulting test data and of the appropriateness of these assays.

In cases where, based on the available genotoxicity studies, it is not possible to conclude on genotoxicity with confidence, i.e. there is high uncertainty (e.g. the absence of the standard or preferred battery of tests, or positive results *in vitro* but negative results in *in vivo* tests), the assessor should take into consideration all available data that may reduce the uncertainty. Data that might be considered include: studies to elucidate the mode of action of the substance under consideration, information on carcinogenicity testing, reproductive toxicity testing, data from toxicokinetic studies, read-across from structurally related substances and predictions from quantitative structure–activity relationship (QSAR) models within their applicability domain. Other data that could be considered are

reliable data from non-standard tests/endpoints (e.g. DNA adducts). If after assessing all lines of evidence and completing all steps of the WoE approach, it is still not possible to conclude on the genotoxicity, additional data would be needed to reduce the uncertainty.

3.3.3. Establishing health-based guidance values

Taking all available evidence into account, if the overall evaluation leaves no concern for genotoxicity *in vivo*, a HBGV may be established.

If, based on the overall assessment, concern for genotoxicity remains, establishing an HBGV is not considered appropriate. However, Chapter 8.1 of the SC Opinion on genotoxicity testing strategies (EFSA Scientific Committee, 2011) describes some circumstances under which genotoxicity might occur only at doses resulting in saturation of detoxification pathways or in cases of substances that interact with molecular targets other than DNA (e.g. DNA polymerases, topoisomerases and spindle proteins). In such cases, provided robust data on the underlying mode of action are available and taking into account all other relevant information, establishing a HBGV might be possible.

In cases where an HBGV had been previously established for a substance and cannot be confirmed due to newly identified concerns about genotoxicity, it is up to risk managers to decide on the regulatory status of the substance during the transitional time that new data are generated.

4. Conclusions and recommendations

Question 1:

Regarding the suitability of the UDS to follow-up positive results in *in vitro* gene mutation tests, the SC concluded that, taking into consideration the current EU legislation and guidance documents, this question should be addressed in both a retrospective and a prospective way.

- For future assessments (new submissions), the EFSA SC is not aware of situations or chemical classes that can be identified in which the UDS could be considered preferable to the TGR or comet assay. Therefore, it is recommended no longer performing the UDS test.
- For re-assessment, in cases of already existing UDS data as a follow-up of a positive *in vitro* mutation test, there might be positive or negative results:
  - Test results may be considered as adequate to assess genotoxic potential only in cases with positive results.
  - If the outcome of the UDS is negative, the reliability and significance of results should be carefully evaluated in a WoE approach, taking into account all available information on mode of action (e.g. the type of DNA damage), metabolism, toxicokinetics, etc., before deciding whether more sensitive tests such as TGR or *in vivo* comet assay would be needed to complete the assessment

Question 2:

Regarding the adequacy to demonstrate target tissue exposure in *in vivo* studies, particularly in the mammalian erythrocyte MN test, the SC concluded that, taking into consideration the current EU legislation and guidance documents, this question should be addressed in lines of evidence of bone marrow exposure.

a) Toxicity to the bone marrow in itself may provide sufficient evidence of bone marrow exposure to allow concluding on the validity of a negative outcome of a study based on the *in vivo* mammalian erythrocyte MN assay. All other lines of evidence of bone marrow exposure should be assessed within a WoE approach to decide whether they might provide sufficient reassurance of a valid negative test result.
b) In the absence of information to confirm bone marrow exposure, further testing would be required to conclude that the substance is not genotoxic (EFSA Scientific Committee, 2011).

Question 3:

Regarding the assessment of the genotoxic potential of substances in a WoE approach and the consequent setting of HBGVs for use in human health risk assessment, the SC concluded that:

a) In case it is not possible to conclude on genotoxicity with confidence, the assessor may, in a second step, take into consideration all available data that may assist in reducing the

uncertainty, including studies on mode of action, read-across from structurally related substances and predictions from QSAR models within their applicability domain. Information on carcinogenicity testing and reproductive toxicity testing, and other information such as ADME may also assist in reducing the uncertainty. If it is still not possible to conclude on genotoxicity, additional data would be needed to reduce the uncertainty.

b) If the overall evaluation, taking all of the available evidence into account, leaves no concerns for genotoxicity, a HBGV value may be established. However, if based on the overall assessment, concerns for genotoxicity remain, establishment of a health-based guidance value is considered inappropriate. In cases where a HBGV had been previously established for a substance and cannot be confirmed due to newly identified concerns about genotoxicity, then new data might be needed to clarify the concerns.

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Abbreviations

ADME study	Absorption, Distribution, Metabolism, and Excretion study
Ames test	bacterial reverse mutation test (OECD TG 471)
CA	chromosomal aberration
CAS	Chemical Abstracts Service
CNS	central nervous system
ECVAM	European Centre for the Validation of Alternative Methods
ECHA	European Chemicals Agency
EU-RL	EU reference laboratory
GLP	Good Laboratory Practices
HBGV	health-based guidance value
Hprt	hypoxanthine-guanine phosphoribosyl transferase (gene mutation test using the Hprt gene, described in OECD TG 476)
MLA	mouse lymphoma assay (mammalian gene mutation test using the thymidine kinase (TK) gene, described in OECD TG 490)
MN	micronucleus test
NCE	normochromatic erythrocytes, i.e. mature erythrocytes
NTP	National Toxicology Program
OECD	Organisation for the Economic Cooperation and Development
PCE	polychromatic erythrocytes, i.e. immature erythrocytes
TERA	transparency and engagement in risk assessment
TG	Test Guideline
TGR	transgenic rodent assay (OECD TG 488)
UDS	unscheduled DNA synthesis test (in vivo – OECD TG 486)
WoE	weight-of-evidence approach
Xprt	xanthine-guanine phosphoribosyl transferase transgene (gpt). Gene mutation test using the xprt gene, (OECD TG 476)

Appendix A – Analysis of the sensitivity of UDS, TGR and comet assays to detect carcinogens

Analysis of EU reference laboratory (EU-RL) ECVAM database

This analysis was performed using data from the EURL ECVAM database (https://eurl-ecvam.jrc.ec.europa.eu/databases/genotoxicitycarcinogenicity-db). A rigorous methodology and defined criteria were applied for the selection and analysis of the data (Kirkland et al., 2014).

The database includes:

- Data for 726 Ames-positive chemicals from different sources: regulatory agencies, industry and literature databases covering different sectors (industrial chemicals, cosmetics ingredients, plant protection and pharmaceutical products).
- Only chemicals with valid *in vitro* and *in vivo* results for the genotoxicity endpoints and/or for carcinogenicity.
- Only chemicals with a known chemical identity (structure, purity, molecular weight, CAS number).
- Combinations into single entries of free bases and respective simple acid salts or R- and S-isomers for those chemicals where a similar behaviour was expected and/or proven.
- Data for the following tests: *in vitro* tests (Ames, gene mutation using TK gene or gene mutation using Hprt/xprt genes', micronucleus (MN), chromosome aberration (CA)); *in vivo* tests (MN, CA, UDS, transgenic models, DNA breakage (comet and alkaline elution assay)); rodent carcinogenicity.

Criteria for 'overall calls' within the database

- 'Overall calls' were defined for each genotoxicity assay *in vitro* and *in vivo* and carcinogenicity by following defined criteria for the reliability of each study and quality of data for those chemicals appearing in more than one source with different calls. Four categories were considered: (+), (–), (E) and (I). Where information was missing, even for those chemicals with one single data entry, scientific literature was consulted.
- Overall calls were made for carcinogenicity as follows:
  - A positive (+) call was assigned if a positive response was reported in at least one sex of either rats or mice.
  - An equivocal (E) call was assigned if the substance was tested in both sexes of rats and mice and at least one equivocal call was noted, and the other calls were negative. A response is considered as equivocal when it is weak or not reproduced between experiments or between laboratories.
  - A negative (–) call was assigned if the substance was tested in both sexes of rats and mice and all four groups yielded negative responses.
  - An inadequate, and therefore inconclusive (I), call was assigned if the substance was tested in both sexes of rats and mice and the results in at least one of the groups was considered to be compromised because of inadequate dosing (too low), excess mortality, or a concurrent infection, and the other groups produced either negative or equivocal responses. If a substance was tested only in rats or mice and was not carcinogenic, that result was also considered inconclusive. Inconclusive (I) calls were considered 'no valid data' and not included in the data analyses (see below).
- In arriving at overall calls for the various genotoxicity endpoints *in vitro* and *in vivo*, the quality of the study, robustness of the protocol and quality of the data, where available, were taken into account. For example, negative results from a recent Good Laboratory Practices (GLP) study conducted to current guidelines was considered more meaningful than a negative result from an old study that did not comply with current guidelines. Where conflicting results were reported in the different databases, the numbers of + and – calls were not considered as important as the quality and robustness of the tests, and whether the results had been obtained in different studies or from different publications, i.e. independent confirmations were more important than the same study result (e.g. from National Toxicology Program (NTP)) reported in different databases.

- For overall calls for each chemical and each genotoxicity endpoint *in vivo*, the following criteria were adopted[9]:
  - An overall positive (+) call for the *in vivo* genotoxicity studies was given if there was clear evidence of a positive response from a single study (rats or mice, males or females). If there was clear weight-of-evidence from more than one study, or if a substance was positive in one species or sex and negative in the other, it was assigned a positive call. In the case of the latter, if two studies gave different results, but it was clear that systemic exposures were greater in the positive than in the negative study, an overall call of + was given.
  - An overall negative (−) call for *in vivo* studies was given when all the requirements of the current OECD guidelines or recommended best practices were fulfilled and there was no evidence of a positive or equivocal response. A negative call was made for *in vivo* genotoxicity studies only if there was evidence that the test substance reached the target tissue, otherwise it was considered inconclusive (see below).
  - An overall equivocal (E) call was given if results were ambiguous, doubtful, questionable, or inconsistent (e.g. a positive and a negative test) within a study, or if a dose-related increase in effects was noted close to the borderline of biological significance, but they were not biologically and/or statistically significant and no independent repeat experiment was done to check the response and produce a clear conclusion. An 'E' call was also used where there were both positive and negative findings across different studies of apparent equal validity, and where the weight-of-evidence did not allow a clear positive or negative overall outcome to be concluded.
  - An overall inconclusive (I) call was given in cases of negative or unclear results, where no firm conclusion could be made in terms of meeting the requirements of the current OECD guidelines or recommended best practices.

For the analysis performed and reported below, the equivocal results were considered as positive call (precautionary principle). Inconclusive results have not been considered.

The sensitivity, specificity and concordance used to describe the performance of genotoxicity tests to predict rodent carcinogenicity were calculated as follow (EFSA J. 2011):

	Carcinogens	**Non-carcinogens**
Genotoxicity positive	A	B
Genotoxicity negative	C	D

Sensitivity	% correctly identified carcinogens	$A/(A + C) \times 100$
Specificity	% correctly identified non-carcinogens	$D/(B + D) \times 100$
Concordance	% correctly identified carcinogens and non-carcinogens	$(A + D)/(A + B + C + D) \times 100$

[9] Only criteria for the *in vivo* overall call are reported since relevant for the analysis made.

Appendix B – Analysis of sensitivity of UDS, transgenics and comet assays to detect carcinogens – results

Table B.1: Ames-positive chemicals which were tested both in the UDS and for carcinogenicity

UDS	Carcinogenicity		Total
	Positive	Negative	
Positive	46	2	48
Negative	31	8	39
Total	77	10	87
	59.70%	80.00%	
	Sensitivity	Specificity	

UDS: unscheduled DNA synthesis.

Table B.2: Ames-positive chemicals which were tested both in the TGR and for carcinogenicity

Transgenic	Carcinogenicity		Total
	Positive	Negative	
Positive	67	0	67
Negative	9	1	10
Total	76	1	77
	88.20%	100.00%	
	Sensitivity	Specificity	

Table B.3: Ames-positive chemicals which were tested both in the comet and for carcinogenicity

Comet	Carcinogenicity		Total
	Positive	Negative	
Positive	52	4*	56
Negative	5	5	10
Total	57	9	66
	91.20%	55.50%	
	Sensitivity	Specificity	

*: Glyoxal (alkaline elution) included.

Table B.4: Sensitivity (prediction of carcinogenicity) of 19 carcinogens of the ECVAM database that were all tested in UDS, TGR and comet assay

	Positive/carcinogens (sensitivity)
UDS	13/19 (68%)
TGR	14/19 (74%)
Comet	18/19 (95%)

UDS: unscheduled DNA synthesis; TGR: transgenic rodent.

Table B.5: Ames positive carcinogens tested in UDS (positive), comet and/or TGR assays. The results of the analysis for the prediction of carcinogenicity by the 3 assays are reported in Tables B.1, B.2, B.3 and B.4

Chemical	CAS no.	Ames overall	*In vivo* UDS Overall	*In vivo* MN Overall	*In vivo* CA Overall	Transgenic Overall	*In vivo* DNA damage Overall	CARC Overall
Acetochlor	34256-82-1	+	+		–		–	+
2-Acetylaminofluorene	53-96-3	+	+	+	+	+	+	+
Acid blue 9	2650-18-2	+	Weak +					
4-Aminobiphenyl (free base + HCL salt)	92-67-1/2113-61-3	+	+	+	+	+		+
2-Amino-1-methyl-6-phenylimidazo(4,5-b) pyridine (PhiP – free base and HCl salt)	105650-23-5	+	+		–	+		+
Azaserine	115-02-6	+	+	+				+
Azoxymethane	25843-45-2	+	+					+
Benzidine (free base and 2HCl salt)	92-87-5/531-85-1	+	+	+	+			+
Bis(dimethylamino)benzophenone (Michler's ketone)	90-94-8	+	+					+
3-Chloro-4-(dichloromethyl)-5-hydroxy-2 (5*H*)-furanone (AKA MX)	77439-76-0	+	+	+		–	+	+
C.I. Direct black 38	1937-37-7	+	+	+				+
C.I. Direct blue 53	314-13-6	+	+				–	
C.I. Direct brown 95	16071-86-6	+	+					+
C.I. Solvent yellow 3 (*o*-aminoazotoluene)	97-56-3	+	+			+		+
6-[*p*-(*N*-β-Cyanoethyl-*N*-methylamino) phenyl-azo]benzthiazol	92887-88-2	+	+					
2,4-Diaminotoluene (free base and 2HCl salt)	95-80-7/636-23-7	+	+	–		+	+	+
1,2-Dibromo-3-chloropropane	96-12-8	+	+	+	+	+		+
1,2-Dibromoethane	106-93-4	+	+	E	–	–	+	+
3,3′-Dichlorobenzidine	91-94-1	+	+	E	+		+	+
Diepoxybutane/1,2,3,4-diepoxybutane/ 1,2,3,4-DL-diepoxybutane	1464-53-5/298-18-0/30419-67-1	+	+	+		–	+	+
N,*N*-Dimethyl-4-aminoazobenzene	60-11-7	+	+	–			+	+
4-Dimethylaminoazobenzeneazo-1-naphthalene	607-59-0	+	+					

Chemical	CAS no.	Ames overall	*In vivo* UDS Overall	*In vivo* MN Overall	*In vivo* CA Overall	Transgenic Overall	*In vivo* DNA damage Overall	CARC Overall
4-Dimethylaminoazobenzeneazo-2-naphthalene	613-65-0	+	+					
5-(*p*-Dimethylaminophenylazo) benzothiazole	18463-90-6	+	+			+		
6-(*p*-Dimethylaminophenylazo)benzthiazole	18463-85-9	+	+			+		+
5-*p*-Dimethylaminophenylazoindazole	17309-86-3	+	+					
4-Dimethylaminostilbene	838-95-9	+	+					
5,9-Dimethyldibenzo[c,g]carbazole	88193-04-8	+	+			+	**Weak +**	+
1,6-Dinitropyrene	42397-64-8	+	+			+		+
2,4-Dinitrotoluene	121-14-2	+	+	–				+
2,6-Dinitrotoluene	606-20-2	+	+					+
Dinitrotoluene, technical grade	25321-14-6	+	+					+
Epichlorhydrin	106-89-8	+	+	–	**E**			+
Glycidamide	5694-00-8	+	+	+		+		
3′-Methyl-4-dimethylaminoazobenzene	55-80-1	+	+					+
Methyl methanesulphonate	66-27-3	+	+	+	+	+	+	+
N-Methyl-*N*′-nitro-*N*-nitrosoguanidine	70-25-7	+	+	+	+	+	+	+
6-Monomethylaminophenylazobenzthiazole	92911-19-8	+	+					
2-Naphthylamine [CGX]	91-59-8	+	+	**E**			+	+
2-Nitrofluorene	607-57-8	+	+					
2-Nitropropane	79-46-9	+	+					+
N-Nitrosodiethylamine (diethylnitrosamine)	55-18-5	+	+	–		+	+	+
N-Nitrosodimethylamine (dimethylnitrosamine)	62-75-9	+	+	+		+	+	+
N-Nitroso-*N*-methylurea	684-93-5	+	+	+	+	+		+
Quinoline	91-22-5	+	+	+	+	+		+
Riddelliine	23246-96-0	+	+	+		+		+

UDS: unscheduled DNA synthesis; MN: micronucleus test; CA: chromosomal aberration.
E = equivocal result, when response is weak or not reproduced between experiments or between laboratories.
I = inconclusive or (more usually) inadequately tested (e.g. not tested both with and without S9, insufficient concentrations, insufficient toxicity, etc.

Table B.6: Outcome of testing of Ames positive chemicals in UDS *in vivo* and other *in vivo* genotoxicity assays

UDS	*In vivo* genotoxicity*		Total
	Positive	Negative	
Positive	40	4	44
Negative	26	41	67
Total	66	45	111
Overall concordance			73.00%

*: In either mammalian erythrocyte MN, CA, TGR or comet assays.
UDS: unscheduled DNA synthesis.

Appendix C – Examples of endpoints which might contribute to the weight-of-evidence assessment

Clinical signs of toxicity

General clinical observations (cage-side) include certain CNS-related signs (e.g. reduction of spontaneous activity, ataxia, sedation, staggering, salivation, abdominal position). Detailed clinical observations (outside the cage), which should be made in OECD test guideline studies such as the 28-day and 90-day studies (OECD TG 407, TG 408, TG 409) and the chronic toxicity studies (OECD TG 452, TG 453), should include, e.g. changes in autonomic activity (lacrimation, piloerection, pupil size and unusual respiratory pattern), in gait, posture and response to handling as well as the presence of clonic or tonic movements, stereotypies or bizarre behaviour. Such clinical signs would usually occur only if the test substance is systemically bioavailable and are thus lines of evidence of systemic bioavailability, and consequently, lines of evidence of bone marrow exposure.

Haematology

The following haematological examinations should be made in OECD test guideline studies such as the 28-day and 90-day studies (OECD TG 407, TG 408, TG 409) and the chronic toxicity studies (OECD TG 452, TG 453): haematocrit, haemoglobin concentration, erythrocyte count, total and differential leukocyte count, platelet count and a measure of blood clotting time/potential. Other haematology parameters such as Heinz bodies or other atypical erythrocyte morphology or methaemoglobin may be measured as appropriate depending on the toxicity of the substance. Haematological changes observed in a repeated-dose toxicity study such as e.g. decreased erythrocyte count, haemoglobin concentration, haematocrit, leukocyte count, if evaluated as being test-substance related and toxicologically significant, are lines of evidence of systemic bioavailability, and consequently, lines of evidence of bone marrow exposure.

Clinical biochemistry

Clinical biochemistry determinations to investigate major toxic effects in tissues and, specifically, effects on kidney and liver should be made in OECD test guideline studies such as the 28-day and 90-day studies (OECD TG 407, TG 408, TG 409) and the chronic toxicity studies (OECD TG 452, TG 453) differ slightly between the different test guidelines. In general, the following parameters should be determined: sodium, potassium, glucose, total cholesterol, urea, creatinine, total protein and albumin, at least two enzymes indicative of hepatocellular effects (such as alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, γ-glutamyl transpeptidase and glutamate dehydrogenase), bile acids, bilirubin. Other clinical chemistry parameters such as fasting triglycerides, specific hormones and cholinesterase may be measured as appropriate, depending on the toxicity of the substance (chemicals in certain classes or on a case-by-case basis). Changes in clinical biochemistry parameters observed in a repeated-dose toxicity study, if evaluated as being test-substance related and toxicologically significant, are lines of evidence of systemic bioavailability, and consequently, lines of evidence of bone marrow exposure.

Urinalysis

The following urinalysis determinations should be made in OECD test guideline studies such as the 90-day non-rodent study (OECD TG 409) and the chronic toxicity studies (OECD TG 452, TG 453): appearance, volume, osmolality or specific gravity, pH, protein, and glucose. These determinations are optional in the 28-day and 90-day rodent studies. Changes in urinalysis parameters observed in a repeated-dose toxicity study, if evaluated as being test-substance related and toxicologically significant, are line of evidence of systemic bioavailability, and consequently, lines of evidence of bone marrow exposure.

Histopathology of organs and tissues

Several organs and tissues should be examined histopathologically in OECD test guideline studies such as the 28-day and 90-day studies (OECD TG 407, TG 408, TG 409) and the chronic toxicity studies (OECD TG 452, TG 453). In general, the following systemically exposed organs and tissues should be examined: brain, spinal cord, liver, kidneys, adrenals, spleen, heart, thymus, thyroid, gonads

(testis and ovaries), accessory sex organs (uterus and cervix, epididymides, prostate, seminal vesicles), urinary bladder, lymph nodes, peripheral nerve, skeletal muscle, bone marrow. In the longer duration studies (90-day and chronic), the following additional systemically exposed organs and tissues should be examined: pancreas, mammary gland, pituitary, parathyroid, aorta, gall bladder (mouse). A histopathological change in any of these organs or tissues observed in a repeated-dose toxicity study, if evaluated as being test-substance related, is sufficient evidence of systemic bioavailability, and consequently, lines of evidence of bone marrow exposure.