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IWGT report on quantitative approaches to genotoxicity risk assessment II. Use of point-of-departure (PoD) metrics in defining acceptable exposure limits and assessing human risk☆

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ABSTRACT

This is the second of two reports from the International Workshops on Genotoxicity Testing (IWGT) Working Group on Quantitative Approaches to Genetic Toxicology Risk Assessment (the QWG). The first report summarized the discussions and recommendations of the QWG related to the need for quantitative dose–response analysis of genetic toxicology data, the existence and appropriate evaluation of threshold responses, and methods to analyze exposure-response relationships and derive points of departure (PoDs) from which acceptable exposure levels could be determined. This report summarizes the QWG discussions and recommendations regarding appropriate approaches to evaluate exposure-related risks of genotoxic damage, including extrapolation below identified PoDs and across test systems and species. Recommendations include the selection of appropriate genetic endpoints and target tissues, uncertainty factors and extrapolation methods to be considered, the importance and use of information on mode of action, toxicokinetics, metabolism, and exposure biomarkers when using quantitative exposure-response data to determine acceptable exposure levels in human populations or to assess the risk associated with known or anticipated exposures. The empirical relationship between genetic damage (mutation and chromosomal aberration) and cancer in animal models was also examined. It was concluded that there is a general correlation between cancer induction and mutagenic and/or clastogenic damage for agents thought to act *via* a genotoxic mechanism, but that the correlation is limited due to an inadequate number of cases in which mutation and cancer can be compared at a sufficient number of doses in the same target tissues of the same species and strain exposed under directly comparable routes and experimental protocols.

☆ The opinions and recommendations expressed in this publication are those of the authors, and do not necessarily reflect those of the institutions with which they may be affiliated.

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1. Introduction

A general framework for the assessment of the risk posed by exposures to genotoxic agents has been defined previously by five working groups established by a joint program of the United Nations Environmental Program (UNEP) and the International Commission for Protection against Environmental Mutagens and Carcinogens (ICPEMC) [1]. The key elements of this framework are (1) hazard assessment, (2) assessment of exposure to genotoxic substances, (3) methods for dose and effect assessment, (4) risk characterization strategies for genotoxic environmental agents, and (5) monitoring environmental genotoxicants. The Working Group on Quantitative Approaches to Genetic Toxicology Risk Assessment (QWG), established by the International Workshops on Genotoxicity Testing (IWGT), has extended those recommendations based on discussions during 2012–2014 and at an IWGT meeting in Foz do Iguaçu, Brazil October 31-November 2, 2013. In a companion report of the IWGT QWG [2] we have summarized the discussions, consensus points, and recommendations regarding methods for the analysis of genotoxicity dose–response information and for establishing points of departure (PoDs) from which exposure-related risk can be estimated. This report summarizes the QWG discussions and recommendations related to extrapolation from the PoD in order to establish acceptable human exposure levels or to determine the exposure-related risk of genetic damage.

1.1. Extrapolation below the PoD

Once a PoD for a relevant response in a test system and tissue or cell type relevant to human health has been established, it is necessary to select and apply appropriate extrapolation method(s) and uncertainty factors (also referred to as safety factors or adjustment factors, depending on regulatory application or context of use). The choice of appropriate methods and uncertainty factors depends on knowledge of the mechanisms that determine the dose–response relationship, and therefore information about the mode of action is essential if uncertainty is to be minimized. For example, some non-DNA-reactive mechanisms are expected to have a threshold of exposure below which there is no biological effect (*e.g.* [3–7]). When evidence supporting the mechanism is sufficient, a relatively small margin of exposure below a minimal effect level may be acceptable. On the other hand, DNA-reactive genotoxicants have often been considered to have a finite risk at any dose (*e.g.* [7,8]), and therefore, linear extrapolation below the PoD is often used. Thus, information on the mode of action of the agent under consideration is an important determinant of the method of extrapolating from the PoD to an acceptable exposure level below the range of measurable data. Examples of the types of mechanistic information that have been used by authoritative bodies to support the choice of methodology are presented in Table 1.

While knowledge of mode of action can inform decisions on appropriate methods of extrapolation from the PoD to levels below the measurable range of increases over background rates, the uncertainty factors and level of conservatism applied are part of the risk management decisions and depend on the nature of exposure and the specific regulatory context that applies to the agent under consideration. Therefore these decisions are based primarily on scientific information but are also impacted by legal and policy considerations (*e.g.*, see [21]).

1.2. Extrapolation from in vivo data

Because of the high relevance of *in vivo* animal data to human risk estimation, and the difficulty of extrapolating directly from *in vitro* cellular data to human risk, *in vivo* data are generally given greater weight than *in vitro* results in the quantitative risk estimation process. When performing a risk assessment, the most relevant animal models (species, and target tissues) and genetic endpoints must be selected to determine a suitable PoD (as discussed in the previous report of the QWG [2]). These choices should be based on the adverse effects being evaluated, knowledge of the mechanism of action, potential target tissues, and relevance of animal models to the type(s) of human exposure anticipated.

Because effects vary among different tissues in a given animal model, the selection of appropriate tissues for quantitative dose–response analysis is critical. Also critical is the choice of endpoints to be used. The genotoxic endpoint selected for analysis must reflect the information available from the hazard identification phase of the risk assessment, including the type of damage determined to be characteristic of the agent under study (*e.g.*, gene mutations, DNA damage, chromosomal damage, *etc.*) and of the expected health effect in humans. Further, this genetic endpoint should be consistent with those that have been identified as key events in an adverse outcome pathway leading from exposure to disease [22–24]. The greatest weight should be given to those endpoints that are directly related to human diseases, such as gene mutations, structural chromosomal aberrations, and aneuploidy (see *e.g.* [25]). The appropriate endpoint should be determined in the tissue that is the expected target site of action and/or those with the highest expected exposure to the agent under consideration and potential active metabolites. Gene mutations can be assessed in virtually any tissue using transgenic animal models (*e.g.* [26–28]), while cytogenetic analysis is currently limited to a few tissues in which cells are actively dividing, or can be induced to divide, such as bone marrow, circulating or splenic lymphocytes, or to some extent liver (*e.g.* [29]). When appropriate, the micronucleus and Pig-a assays in hematopoietic cells (bone marrow and/or blood) are highly efficient and relatively cost effective to assess chromosomal damage and gene mutations, respectively.

Although hazard screening is generally carried out using a small number of tissues in which assays are easily conducted, the choice of a suitable tissue for quantitative analysis should be based on available information on (1) site-specific toxicity, (2) mechanism of toxicity and mechanistic expectations of important target tissues (including carcinogenicity if information is available), (3) distribution and metabolism of the test agent in both the animal model system and, if known, in humans, (4) exposure or accumulation of the chemical and its relevant metabolites, (5) cell proliferation, and potentially (6) the ability to repair the DNA damage induced by the agent of interest. The interplay of these parameters will determine not only the target tissues and nature of the toxic and/or genotoxic effects, but also the dose response relationships of the key endpoints of concern, and therefore the determination of the appropriate PoD within a relevant animal model. Other parameters, such as the sensitivity or variability of the background frequency of an endpoint and the number of animals and/or cells evaluated, are also important to consider, especially in regard to the statistical evaluation and sensitivity of the assays employed.

Other assays that measure DNA damage or cellular responses to DNA damage and can be applied to multiple tissues are useful but are given less weight than assays for mutations or chromosomal aberrations because such primary DNA lesions may be repaired before conversion to inheritable DNA changes. Such assays and endpoints include DNA adducts [30], DNA strand breaks or alkali-labile sites measured using techniques such as the comet assay [31], or DNA repair or other damage-response assays (*e.g.* [32]). Assays that use DNA strand breakage as the genotoxic endpoint also have the limitation that DNA strand breaks occur during DNA repair, cell replication, apoptosis, and necrosis, and so strand breakage not directly related to mutation or chromosomal aberration fixation may occur.

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Table 1
Examples of mechanistic information used by authoritative bodies to infer that a non-linear threshold-type dose response occurred or that genotoxicity/carcinogenicity did not occur through a mutagenic or human-relevant mode of action.

Mechanistic information	Example(s)	References
Critical involvement of non-DNA targets	Aneuploidy: benomyl; carbendazim	[9–11]
Contribution of DNA repair mechanisms	Ethylmethane sulfonate	[9,12,13]
Detoxification capacity exceeded	Hydroquinone; paracetamol (acetaminophen)	[9,14]
Disruption of enzymes involved in DNA synthesis or replication	Topoisomerase II inhibitors; anti-metabolites; methotrexate	[9,11]
Chemical reactivity or properties unlikely to occur *in vivo*	Captan; trichloroacetic acid	[14–16,18]
Inadequate uptake or toxicokinetics limiting distribution to target	Chromium III	[14,17]
Mutational spectrum in tumor genes similar to those in untreated animals	Trichloroacetic acid	[14]
Structural similarities to similar threshold-acting chemical	Folpet; captan	[14,18]
Secondary or indirect origin of the observed damage	Oxidative damage; ethylene glycol monobutyl ether	[14,19]
Species and tumor-specific non-genotoxic mode of action	Induction of thyroid follicular cell tumors by inorganic chlorates	[20]

In order to establish exposure limits that minimize human risk of organ toxicity or carcinogenicity, PoD values from animal models in conjunction with uncertainty factors are used (*e.g.* [33–38]). The QWG agreed that the same principles apply to analysis of genotoxicity data. There are currently various approaches in use for setting uncertainty factors when using animal data to assess the risk of chemical toxicity and to determine a PDE (permitted daily exposure), RfD (reference dose), TDI (tolerable daily intake), or ADI (acceptable daily intake) (*e.g.* [34,38–44]). Often, a combination of default uncertainty factors (*e.g.*, up to 10,000-fold or higher adjustments) is used to extrapolate to acceptable human exposure levels when specific data are not available (*e.g.* [33,37,45,46]). These default approaches can be applied when there is no information about pharmacokinetics or actual exposure to the active form of a chemical. Knowledge about exposure, metabolism, and pharmacokinetics can help refine the extrapolation to the human situation, and can often significantly reduce the magnitude of uncertainty factors that must be applied. In the case of the risk assessment conducted after exposure of patients to EMS as a result of contamination of the HIV drug Viracept®, discussed in Appendix 2 [47], determination of the actual exposure in animals and pharmacokinetic modeling for humans using unlabeled and ^{14}C-labeled EMS allowed replacement of the critical uncertainty factors for exposure with experimentally determined values, thereby considerably lowering the overall uncertainty of the risk determination.

The toxicokinetic properties, absorption, distribution, metabolism, and excretion (ADME) of the agent under consideration are critical factors in the risk assessment. Exposure information can be obtained by direct analytical methods that determine levels of the agent under study and its important metabolites in blood and tissues, or by measurement of cellular reaction products such as DNA or protein adducts [48–50]. DNA adducts are formed by reaction of a chemical with DNA [51,52] and can thus be used as a surrogate marker of a biologically effective dose. The stability of a DNA adduct and its consequent conversion into a stable mutation is determined by the cell's DNA repair capacity and the turnover rate of the damaged target cells. The risk of conversion into permanent genetic damage before repair is completed is dependent on many factors, including the nature of the adduct and its position in the DNA as well as the number of induced adducts in a specific tissue and specific factors such as cellular replication rate [48]. Thus, the likelihood that an adduct will be converted to a mutation depends on the type of DNA adduct, its effect on base pairing, and the efficiency and fidelity of DNA repair in a particular tissue or cell type. The characteristics of the DNA adducts and of repair processes often also provide insights into the mechanism of cellular pathology. It is important to note that various types of DNA repair are error-prone, and so not only the potential fixation of a mutation *via* misreading the adducted base but also the potential for errors arising from removal of the adduct or by translesion synthesis may be determinants of the mutational risk [53]. Thus, adduct data can be used to quantify exposures, can contribute to elucidating the biological mechanism of mutagenic and clastogenic events, and/or can be used to quantitatively evaluate dose response relationships.

Methods and analytical tools for adduct identification and/or quantitation have been summarized and reviewed (*e.g.*, [49,54,55]). Such data can be used to identify specific adduct patterns induced by exogenous chemicals and thus contribute to elucidating the biological mechanism and/or used to quantitatively evaluate the dose response relationship. Due to the active removal of DNA adducts by repair mechanisms, a quantitative evaluation needs to take into account the kinetics of these processes. When using DNA adducts to support quantitative risk assessment, it is of course necessary to take into account the level of DNA adducts formed endogenously by normal cellular metabolism, oxidative stress, and daily background exposures [56], as well as the chemical's toxicokinetic properties, including absorption, distribution, metabolism, and excretion (ADME) [48–50,56].

Hemoglobin adducts can also serve as sensitive surrogate markers of "internal" exposure to a chemical [57,58]. In contrast to DNA adducts, hemoglobin adducts are not actively removed from the protein, and therefore their half life depends only on the erythrocyte lifespan if the adduct is chemically stable. For a direct extrapolation from animals to humans based on exposure data from hemoglobin adducts, the species specific lifespan of erythrocytes needs to be taken into consideration (approximately 41, 61 and 122 days in mice, rats and humans respectively [59–61]). The relatively well characterized and long lifespan of the erythrocyte make hemoglobin adducts a sensitive and quantitative indicator of exposure and allows estimation of the area under the (concentration–time) curve (AUC) values determined in toxicokinetic studies [62]. Mechanism-based models using hemoglobin adducts as a measure of AUC have been proposed for cancer risk assessment of genotoxic chemicals [63,64]. However, hemoglobin adducts cannot be used to directly address exposure levels in target tissues or organs other than blood. DNA adducts can be measured directly in the relevant target tissues of concern, but are less useful for AUC calculations as their rates of repair vary and it is more difficult to obtain repeated measures of tissues that can only be accessed invasively. Information from animal experiments on the proportionalities between formation of DNA adducts in different organs and of hemoglobin in blood can be useful for extrapolation to internal tissue dose (or AUC) in different organs when such information is known for a given chemical or class of chemicals.

To illustrate how the considerations discussed above can be addressed experimentally and the results interpreted quantitatively in the context of the risk assessment framework presented, we summarize in Appendix 2 the risk assessment conducted when a large number of patients were exposed to an ethyl methanesulfonate (EMS) contaminant in the drug Viracept®, administered for treatment of human immunodeficiency virus (HIV) infection. Due

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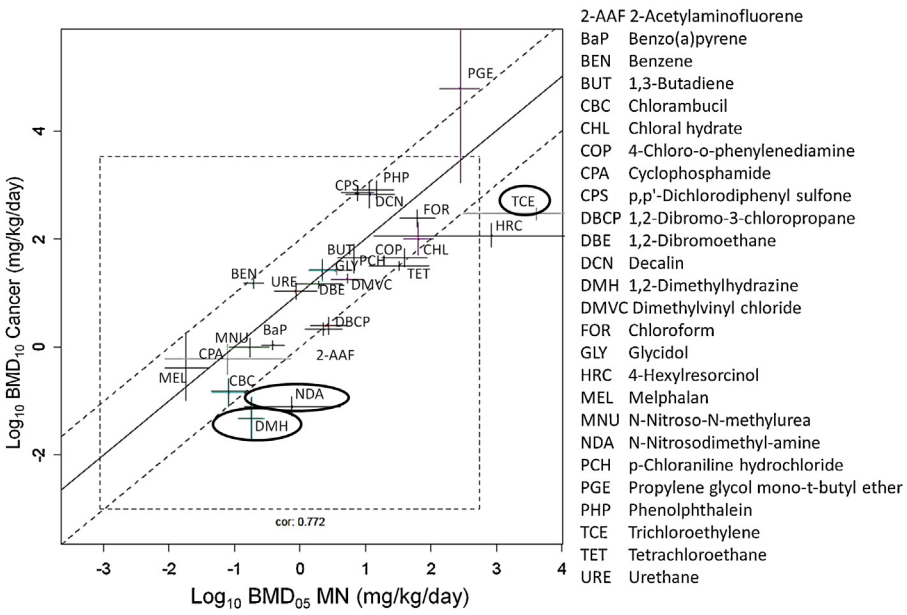

Fig. 1. A plot of BMD_{10} for cancer induction *vs.* BMD_5 for micronucleus induction calculated from 26 cases in which both rodent cancer and micronucleus induction data were available for the same agent. For each chemical the point of crossing indicates the point estimates of the BMD. The corresponding crossing lines indicate the BMDL (low end of line) and the BMDU (high end of line). Thus the total length indicates the statistical uncertainty in the BMD value. The solid diagonal line indicates the point estimates at which the carcinogenic BMD_{10} values are 10-fold higher than the micronucleus BMD_5 values. The upper and lower dashed lines represent 10-fold above and below the solid line. Modified from [66], with permission.

to the highly significant exposure to this DNA-reactive mutagen, it was necessary to determine the risk of genetic damage in the exposed individuals and to determine whether appropriate follow-up health care and possible long-term monitoring for carcinogenic outcome *via* a cancer registry was necessary. The approach taken represents a case of a quantitative risk analysis in which good experimental genotoxicity data combined with mechanistic and exposure information allowed the conclusion of negligible risk at a calculated margin below the derived PoDs in the absence of sufficient carcinogenicity data. The exposed individuals had exposures lower than the derived negligible risk levels, and the responsible regulatory authority concurred that the analysis demonstrated a negligible risk for the exposed population. This example reinforces our conclusion that good exposure and PK information combined with a mechanistic understanding of cellular protective mechanisms allows uncertainty factors to be minimized and can lead to agreement by regulatory authorities on acceptable exposure levels.

1.3. Quantitative correlations between cancer potency, early cancer-related genetic endpoints, and preneoplasia

Given the importance of predicting carcinogenic risk and the known association of genetic damage with cancer induction, the QWG also considered quantitative correlations between carcinogenic and mutagenic potency for chemicals for which there is mechanistic information suggesting that mutagenicity (or clastogenicity) is a key event on the pathway to carcinogenesis. The objective was to determine the extent to which quantitative genotoxicity data, particularly mutagenicity and clastogenicity data, could be used to predict carcinogenic outcomes and potency and to refine cancer risk assessments. The QWG considered two types of correlation: (1) correlation of *in vivo* mutagenic and clastogenic potency with carcinogenic potency without restriction to identical species, strain, sex, or tissue target sites, and (2) correlation of *in vivo* mutagenic and clastogenic potency with carcinogenic potency within a given target tissue in the same species, strain, and sex of laboratory animal exposed by the same route.

1.4. Correlation of in vivo mutagenic and clastogenic response with carcinogenic potency without restriction to identical species, strain, sex, or tissue target sites

The quantitative relationship between gene mutation in transgenic rodent models and micronucleus induction (an index of chromosomal aberrations) in bone marrow (or peripheral blood erythrocytes) with carcinogenic potency has been examined by Hernandez et al. [65,66], using quantitative dose–response analysis of the genotoxicity data. Although these data were, in general, not tissue or strain matched, a remarkably good quantitative correlation with carcinogenic potency was observed.

The comparison of BMDL values calculated using data from micronucleus and transgenic rodent studies *vs.* the carcinogenic $BMDL_{10}$ was examined for 18 compounds [65]. The correlation of carcinogenic with mutagenic/clastogenic potency over five orders of magnitude showed a high degree of correlation on a log-log scale even though genotoxicity data were in most cases from tissues other than the cancer target site (*i.e.*, genotoxicity data were from the tissue in which the tumor arose for only 4 of the compounds used in this analysis, and the route of exposure was different for half of the compounds). In another study, analysis of 26 compounds for which micronucleus and carcinogenic BMDs could be calculated also showed a strong correlation between carcinogenic and genotoxic potencies as estimated by the reciprocal of the BMD [66]. These data from the latter study, shown in Fig. 1, illustrate both the general overall correlation observed and also the factors that limit such comparisons when tissue target site, species, strain, sex, route of administration and other experimental variables differ among the studies being compared.

Fig. 1 shows a clear correlation between the cancer and the micronucleus BMDs, even though the species, strain, sex, dosage route, and tissue target sites for cancer and micronucleus induction were not matched. Given that metabolic activation and tumor induction generally occur in tissues other than bone marrow (the target tissue in the micronucleus assay), and that tumor potency often differs significantly among different tissues and different species/stains of animal, this is a remarkably good correlation and

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suggests that tissue-matched data should give an excellent correlation. The $BMDL_5$ for the genetic toxicity endpoint was lower than the tumor $BMDL_{10}$ in 23/26 (88%) cases. Of course this percentage will be different for different methods for calculating the BMD and different sizes of BMR (See [66] for the method used to generate Fig. 1). Nevertheless the correlation should remain, regardless of how the BMD is defined or calculated. There were only three compounds for which the tumor $BMDL_{10}$ was lower than the $BMDL_5$ for micronucleus induction (Fig. 1). For tetrachloroethylene (TCE), the upper limit of the micronucleus BMD_5 (the $BMDU_5$) is infinite, which is consistent with the micronucleus data not showing a statistically significant dose response. *N*-nitrosodimethylamine (NDA) is genotoxic but is activated to a short-lived reactive intermediate in liver, the site of carcinogenesis, and therefore genotoxicity in bone marrow would not be expected to predict quantitative responses in liver. 1,2-dimethylhydrazine (DMH) is a colon carcinogen that is metabolized in the liver to a conjugate with glucuronic acid. This glucuronide enters the gut both with bile and directly *via* the circulation, and microbial beta-glucuronidase releases the active metabolite which, in turn, alkylates tissue macromolecules [67]. This mechanism of carcinogenic action of DMH in rat colon is not expected to occur in bone marrow.

Thus, considering both the Hernandez et al., 2011 and 2012 reports [65,66], there is a general quantitative correlation between carcinogenic and mutagenic or clastogenic potency for a wide range of agents believed to have a genotoxic mode of cancer induction, but metabolic, toxicokinetic, and distributional properties limit the precision of these potency correlations when comparisons are made between studies that differ in tissue target site, species, strain, sex, dosage route, *etc.*, unless these factors are taken into account. Nonetheless, these results suggest that, in the absence of carcinogenicity data, an estimate of probable cancer potency can be derived from *in vivo* genotoxicity studies.

1.5. Correlation of mutagenic and clastogenic response with carcinogenic outcome in the same target tissue, species, strain, and sex of animal when exposed by the same route

Consideration of the dose–response characteristics of mutagenic and clastogenic responses in the cancer target site (organ/tissue of the species and strain under study) was undertaken to determine if such data could be useful in refining carcinogenesis risk estimates in those cases in which mutagenesis or clastogenesis are key events in the pathway to carcinogenesis. Because the determination of cancer incidence is always limited by the statistical uncertainty associated with a stochastic response in a small number of animals, it was hoped that mutation data and concurrent data on pre-neoplastic lesions, such as glutathione S-transferase (GST)-positive foci in liver, which can readily be determined in a cost-effective manner, could be used to improve the estimates obtained.

A survey of available data on genotoxicity outcomes for chemicals that have undergone cancer bioassays, taking into account that assays with OECD test guidelines are preferred because standardized protocols greatly facilitate comparisons, found that the standardized *in vivo* genotoxicity assay for which data was most often available was the rodent erythrocyte micronucleus assay. However, this assay only provides information about two types of genotoxic damage (structural chromosomal damage and aneuploidy) in the hematopoietic cells of the erythrocytic lineage. Establishment of an OECD guideline for the transgenic rodent mutation assays has resulted in increasing amounts of data on gene mutations using standardized protocols, but the number of cases in which mutagenicity data were available at a sufficient number of doses in the organ/tissue target site of carcinogenesis, and in the same species and strain in both assays, was extremely limited. Likewise, we found only a very limited number of cases in which data on pre-neoplastic lesions were available along with comparable cancer and genotoxicity data in comparable species, strains, tissues, and exposure regimens. In those few cases in which we did identify matched carcinogenicity and genotoxicity data, there were generally not a sufficient number of doses to obtain quantitative dose–response metrics. Thus, we were unable to identify a sufficient number of data sets to assess the quantitative correlation between carcinogenic potency and mutagenesis and clastogenesis within the same target tissue, species, strain, and sex. The dearth of such information was unexpected, and constitutes a very significant need in the field of genetic toxicology and cancer risk assessment.

In Appendix 1 we present a case example that illustrates the type of quantitative analyses and comparisons that we had hoped could be conducted for a range of structurally dissimilar chemicals. This example is that of the carcinogenic and genotoxic responses in the liver of F344 rats exposed to 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx) under comparable study conditions. This is the agent for which we identified the most comprehensive set of genotoxicity and carcinogenicity data under comparable study conditions. In this case, as shown in Fig. 2, early genotoxic events in the pathway to carcinogenesis showed the expected trend of lower PoDs for earlier events in the pathway to disease ($\text{DNA adducts}_{4\text{weeks}} \ll \text{Mutations}_{16\text{weeks}} < \text{GST-positive Foci}_{16\text{–}32\text{weeks}} \ll \text{Cancer}_{56\text{weeks}}$). However, the QWG was unable to identify a sufficient number of additional cases with sufficient data to determine if generalizations could be made. We strongly recommend that additional data of this type should be generated for cases in which genotoxicity is a key step in the carcinogenesis process so that these important relationships can be studied.

2. The role of *in vitro* data

2.1. Mammalian cells

Genotoxicity data from *in vitro* mammalian cell assays are used routinely for hazard identification, mechanistic studies, and as supporting information in the quantitative risk assessment process. The QWG also discussed the feasibility of estimating quantitative *in vivo* risk by extrapolating from *in vitro* data. The 2007 National Research Council Report "Toxicity Testing in the 21st Century: A vision and a strategy" [68,69] recommends development of an integrated, toxicity pathway-oriented approach based on computational systems biology pathway modeling using *in vitro* cells in combination with reverse dosimetry models to derive acceptable *in vivo* exposure limits. Such approaches are still in an early stage of development and their utility for routine quantitative assessment of hazard and risk remains unclear. One example of an attempt to use such an approach for the analysis of genetic effects is the recent report of Li et al. [70] that describes dose–response modeling of the effects of etoposide on DNA damage and damage-response pathways. To build such models, however, requires extensive data on free concentrations in solution, protein binding, *etc.* Other limitations of using mathematical models of toxicity pathway perturbations for estimating small increases in risks, a major focus of the National Academy document, have been discussed by Crump et al. [71,72].

Various approaches for physiologically based pharmacokinetic (PBPK) modeling to conduct quantitative *in vitro* to *in vivo* extrapolation are currently being investigated [73,74]. Many of these currently focus on specific endpoints such as prediction of metabolism or clearance. Much effort is still needed to improve these approaches before they become routine tools to improve quantitative *in vitro* to *in vivo* extrapolation for genotoxicity. In

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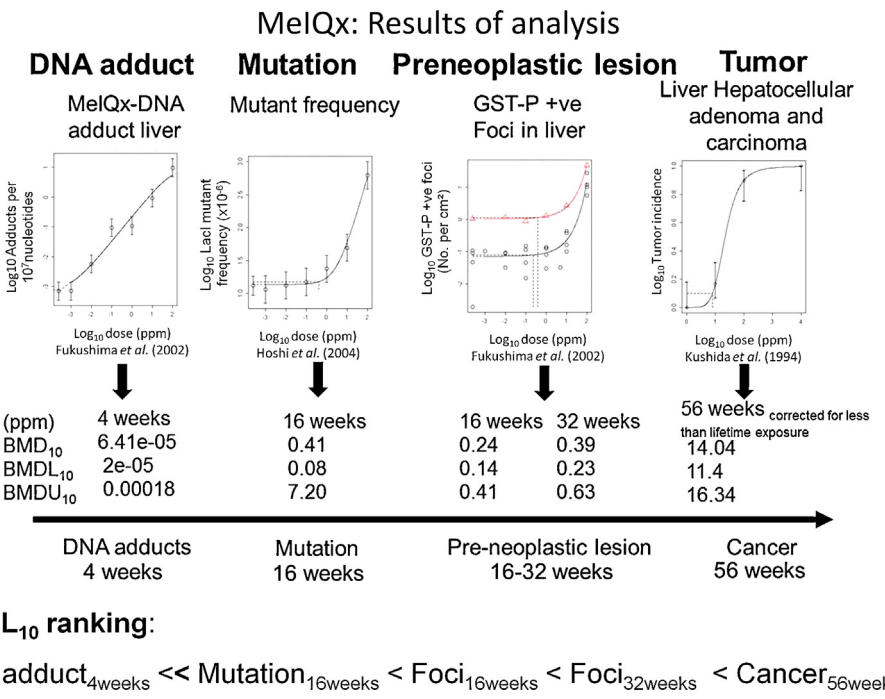

Fig. 2. Dose–response plots and derived BMD values for DNA adducts, mutations, GST-positive foci, and liver hepatocellular adenoma and carcinoma induced in F344 rat liver by exposure to MeIQx.

order to draw a final conclusion, data on the intracellular exposure of chemicals would likely be required to build and apply the models, but such data are rare. Therefore risk assessment based on *in vitro* mammalian cell data should probably be limited to situations with a relatively low risk as identified by hazard screening and exposure assessments, in which *in vivo* metabolism is well characterized and can be properly modeled, and/or by using additional uncertainty factors if extrapolation is made from the BMDLs of *in vitro* experiments to acceptable human exposure.

In general, uncertainty factors would be expected to be related to the degree of relevance of the experimental model to humans, and uncertainty factors would be included resulting in a cumulative factor by which the margin of exposure would be divided. This relationship and the relationship of exposure information in different models are illustrated in Fig. 3.

Although there are clearly uncertainties when quantitatively extrapolating *in vitro* data to estimate human *in vivo* risk, *in vitro* studies can be useful when knowledge of mechanism, metabolism, and exposure permits quantitative extrapolation or comparison. For example, *in vitro* data may allow the estimation of the *in vivo* concentration of an unbound direct-acting DNA-reactive agent that would be expected to elicit a defined BMR (*e.g.*, obtained by extrapolating data from a PoD to the benchmark concentration (BMC) at the desired BMR), and in the absence of *in vivo* genotoxicity data this may permit estimation of an acceptable *in vivo* dose associated with minimal risk of genotoxic damage (provided that *in vivo* data that relates dose to blood and/or tissue concentrations is available). Another use would be to use a defined BMD or BMC to rank potencies of related compounds in a series. And importantly, as increased knowledge and experience with regard to analysis of responses in adverse outcome pathways and *in vitro* measurement and prediction of exposure to chemical species responsible for pathway perturbations continue to accumulate, it is expected that the utility of these approaches for quantitative predications will continue to increase.

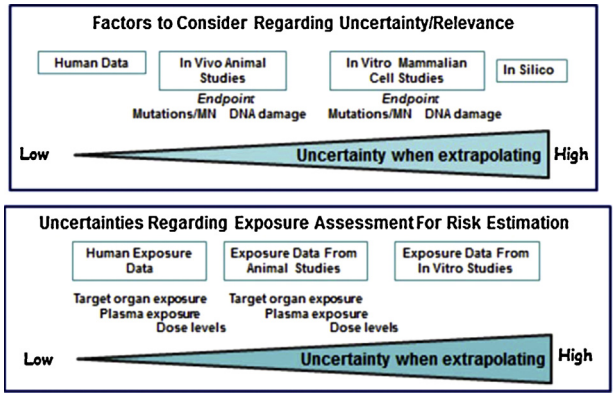

Fig. 3. Uncertainty related to estimation of human risk.

2.2. Bacterial mutagenicity assays

The question of whether or not a quantitative relationship exists between mutagenic potency in the *Salmonella* (SAL) based Ames test [75,76] and carcinogenic potency was a matter of scientific debate in the late 1970s. Early reports showed a high qualitative positive predictivity (>90%) of SAL results for rodent carcinogenicity [76,77] but did not address whether there was a quantitative correlation with carcinogenic potency. When a larger number of chemicals with diverse modes of action had been evaluated, even the qualitative correlation between SAL and carcinogenic potency in rats was found to be much lower than originally thought [78–82]. From a quantitative perspective, Meselson and Russel [83] for example, initially found a nearly perfect linear relationship between carcinogenic potency in rodents and mutagenic potency in *Salmonella* for 10 of the 14 chemicals studied, but this quantitative predictivity was considerably lower when a broader set of chemical classes was investigated [84–86]. The QWG recognizes that bacterial mutagenicity assays are useful for the evaluation of reactive potential and ability to interact with DNA, that many documented human carcinogens are positive in

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the Ames assay, and that they are therefore important components of hazard evaluation and mechanistic studies. However, the QWG noted that while bacterial assays can be useful for rank ordering the mutagenic potency within structurally similar compounds, bacterial physiology and anatomy is too different from mammals and humans to support their use for quantitative extrapolation to human risk.

2.3. Metabolic activation in in vitro studies

One factor that limits quantitative extrapolation from *in vitro* systems to *in vivo* effects is the fact that adverse interactions of many mutagenic agents with genetic material only occurs following *in vivo* metabolic conversion into reactive metabolites. Early studies showed that *in vivo* mammalian metabolic conversion of mutagenic carcinogens such as dimethylnitrosamine and 2-acetylaminofluorene to highly reactive electrophiles is responsible for their ability to readily attack DNA (*e.g.* [87]. It was then shown that other classes of mutagenic compounds, including nitrosamines, PAHs, and aromatic amines, are converted to reactive metabolites *via* oxidative hepatic metabolism. In the early 1970s, Malling first suggested using rodent hepatic preparations in *in vitro* bacterial mutagenicity assays to simulate mammalian hepatic metabolism [88]. The use of an exogenous source of metabolic enzymes such as the post-mitochondrial supernatant (PMS) of homogenized rat livers (*i.e.*, referred to as rat liver S9 when the $9000 \times g$ supernatant fraction is employed) from rats previously treated with Ah-receptor agonists such as Aroclor-1254 to increase metabolic activity was then shown to enable demonstration of *in vitro* mutagenic activity for a wide variety of chemical carcinogens [76,89]. Hepatic preparations from other species or strains of animals (*e.g.*, B6C3F1 mouse, F344 rat, Golden Syrian Hamster) and/or alternative tissues or inducers (*e.g.*, phenobarbital/5,6-benzoflavone, ethanol) are now commercially available and are frequently used ([90]; www.moltox.com). A NADPH generating system based on glucose-6-phosphate dehydrogenase, and assay concentrations of hepatic S9 ranging from 1 to 10% v/v (typically 1–2% v/v) are most commonly used. However, although mutagenic activity of many chemical carcinogens can be demonstrated in *in vitro* systems, quantitative extrapolation to *in vivo* carcinogenic potency remains problematic due to the complexity of the metabolic pathways involved.

The utility of human hepatic preparations for the *in vitro* activation of mutagens has been evaluated, and differences in the activity of oxidative hepatic enzymes (*e.g.*, P450 1A1) between human preparations and those routinely employed for *in vitro* genotoxicity assessment have been noted (*i.e.*, Aroclor induced rat liver).[1] Analyses of enzymatic activity data from commercial preparations indicate that average human hepatic levels of EROD and MROD activity (*i.e.*, P450 1A1 and 1A2) are approximately 100 pmols min^{-1} mg $protein^{-1}$ and average levels of BROD activity are approximately 45 pmols min^{-1} mg $protein^{-1}$ (P450 2B1 and 3A), and that the distribution of P450 isozyme activity in humans is highly positively skewed. Some pooled preparations showed EROD, MROD and BROD activities as high as 686, 526 and 296 pmols min^{-1} mg protein. In comparison, average EROD and MROD activity levels in hepatic preparations from uninduced Sprague-Dawley rats are approximately 55 and 15 pmols min^{-1} mg $protein^{-1}$, respectively, with average BROD levels approximately 75 pmols min^{-1} mg $protein^{-1}$. Thus, human hepatic capacity of some P450-mediated oxidation reactions is similar to that expected for uninduced Sprague-Dawley rat; however, Aroclor induction results in increases in EROD and MROD of 100-fold or more, and approximately 30-fold increases in BROD and PROD activity levels (*i.e.*, 2B1, 3B2 and 3A). Thus, the use of induced rat liver S9 adds an element of conservatism because of the large increases in activities that are induced. Induction with other Ah-receptor agonists (*e.g.*, phenobarbital/5,6-benzoflavone) show similar increases in the activity of hepatic P450 isozymes, and relative differences in the capacity for *in vitro* oxidative metabolism between humans and rodents. However, such differences among different inducers add to the complexity of effectively using *in vitro* genetic toxicity assessment data for quantitative evaluation of human risk.

Although exogenous metabolic activation mixtures containing the popular Aroclor-induced rat liver S9 (or similar) have proved to be highly effective for the detection of bacterial mutagens, a number of factors hamper effective use of S9-containing activation systems for *in vitro* genotoxicity assessment in mammalian cells. One important factor is that exogenous metabolic activation mixtures containing hepatic S9 have been shown to be highly toxic for mammalian cells (*e.g.*, CHO, TK6, V79, L5178Y, *etc.*) that are routinely employed for genotoxicity testing [91]. Other factors include inadequate knowledge regarding the endogenous metabolic capacity of the cells, the properties of the compound, the optimal level of exogenous metabolic activation, and binding of the test article to S9 proteins.

It is important to note that *in vivo* metabolism involves a complete complement of Phase I and II enzymes for oxidation, reduction, hydrolysis and conjugation, while *in vitro* activation systems contain a restricted subset of Phase I and II enzymes in an activation mixture that, for the most part, contains cofactors that preferentially facilitate cytochrome P450-mediated oxidative reactions. Although the above discussion has focused on *in vitro* simulation of oxidative metabolism for the generation of mutagenic metabolites, many mutagens require other types of enzymatic reactions. For example, diazo compounds and nitroarenes require reductive metabolism to generate arylamine intermediates that can subsequently be converted to hydroxyarylamines *via* P450 1A2 [92–95]. Potent mutagens can be generated by reductive cleavage of benzidine-based diazo compounds, and metabolic activation mixtures containing FMN (flavin mononucleotide) and hepatic S9 from Golden Syrian Hamsters have been employed to improve detection of this class of mutagens [92]. Moreover, enzymes that facilitate conjugation reactions (*e.g.*, sulfotransferase, glutathione-S-transferase, or acetylase) play an important role in the activation of some mutagens and this can also be simulated *in vitro*, although this is not commonly done. Examples include exogenous activation mixtures or transgenic expression systems for the generation of mutagenic metabolites of halomethanes and *N*-hydroxyarylamines [96–98]. These additional enzymatic requirements further complicate the use of *in vitro* mutagenicity data for quantitative assessments of agents that require metabolic activation for activity.

3. Conclusions and recommendations

3.1. General

- The QWG supports the use of *in vivo* genotoxicity dose–response data to determine PoDs to be used, with appropriate extrapolation methods and uncertainty factors, to establish regulatory exposure limits, and, in conjunction with human exposure data, to assess and manage the risk of adverse health effects.

[1] Personal Communication, manuscript in preparation: J.A.Cox, M.Fellows, T.Hashisume, P.A.White. Utility of Human Hepatic S9 for Routine Regulatory Evaluation of Genetic Toxicity.

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3.2. Recommended endpoints for quantitative human risk assessment

- Among types of genotoxicity endpoints, those associated with human disease should be given the most weight when conducting a human risk assessment. These include mutagenicity, clastogenicity, and aneugenicity. Those selected should also be consistent with those that have been identified as key events in an adverse outcome pathway leading from exposure to disease.
- DNA damage assays, such as DNA strand breaks as determined by the comet assay, measurement of DNA adducts, and cellular DNA damage responses can be useful to determine the DNA reactivity of a chemical or the presence of DNA damage, and can be used to demonstrate an absence of strand breakage and therefore reduced potential to induce heritable alterations. However, their utility for quantitative evaluations is limited because the extent to which DNA damage may be repaired before conversion to a permanent genetic alteration is difficult to ascertain. Stable DNA adducts may have a greater potential for conversion to mutations, especially when of a type known to cause base pairing errors. DNA strand breaks occur during DNA repair and during apoptosis and necrosis, and so strand breakage may not always be related directly to the formation of mutations or chromosomal aberrations.

3.3. Recommended test systems, target tissues, and exposure data for human risk assessment

- The tissues chosen to be used in support of risk assessment should be those most likely to be exposed to the highest level of the reactive form of the toxicant under study and/or be a suspected site of biological action. Selection can be based on available information on (1) toxicity, (2) exposure or accumulation of a chemical, including route of exposure, (3) cell proliferation, (4) metabolism, (5) knowledge of mechanism of action, and potentially (6) DNA repair capacity or even (7) information about carcinogenicity.
- Parameters and uncertainty factors to be considered when assessing risk or setting exposure limits include (a) species differences and allometric scaling, (b) differences in absorption, distribution, metabolism and pharmacokinetics, (c) differences in duration of exposure, (d) severity of toxicity endpoint, (e) variability among individuals, (f) uncertainty in PoD or NOEL. When data that permit assessment of these parameters/factors are not available, uncertainty factors should be applied to the predicted acceptable exposure level to account for the absence of data.
- When the actual exposure and pharmacokinetic/metabolism characteristics of the agent under consideration have been determined, uncertainty factors can be replaced with experimentally-determined parameters that may justify higher allowable exposures.
- In addition to analytical determination of plasma and tissue exposures, DNA or hemoglobin adducts are useful metrics of exposure, and DNA adducts are also useful for determination of the mode of genotoxic action.

3.4. Use of mode of action information in quantitative risk assessment

- Mode of action information is very important, and can help select relevant endpoints for study and appropriate extrapolation methods. It can also help identify potential species differences in critical physiological/biological factors that modify risk and impact uncertainty factors, and can determine whether more or less conservative extrapolation methods and uncertainty factors should be applied in the risk assessment.
- The decision whether a linear or non-linear approach should be used to estimate low dose risks should be based on a mode of action understanding. When adequate MoA information is not available, risk management or policy decisions may need to be made based on default assumptions or regulatory policy requirements.
- The entire weight of the evidence, within the broader context of the toxicity, pharmacodynamics, ADME, and toxicokinetics of the chemical, should be considered.
- Carcinogenic potency of agents believed to involve mutation or chromosomal aberration as key events in the carcinogenic process shows a general correlation with the potency of gene mutation induction *in vivo* and micronucleus induction in hematopoietic tissue, even when the data are from different tissues, experimental protocols, and species/sex/strain of animal. It is expected that the strength of this correlation would be improved if mutation and chromosomal aberration data were available from cancer target tissues in the same strain, species, and sex of animals exposed by the same routes using similar protocols, but sufficient data to obtain the quantitative metrics necessary to examine this assumption were not found by the QWG. The QWG recommends that such data should be generated.
- In the case of MeIQx, a genotoxic carcinogen for which good data on the induction of DNA adducts, mutations, GST-positive foci, and tumors in the liver of F344 rats are available, an analysis of the dose–response relationships showed that the quantitative order of induction of these events was: DNA $\text{adducts}_{4\text{weeks}} \ll \text{Mutations}_{16\text{weeks}} < \text{GST-positive Foci}_{16\text{–}32\text{weeks}} \ll \text{Cancer}_{56\text{weeks}}$.

3.5. Use of in vitro data in quantitative human risk assessment

- *In vitro* mammalian cell data may be useful in quantitative risk assessment if exposure to the active form of the agent is known. Differences in bacterial permeability, DNA repair, and metabolism as compared to mammalian cells make quantitative extrapolation from bacteria very difficult, other than for potency ranking within structural classes. Mammalian cell data may also be used for potency ranking among related agents with similar modes of action.
- Factors of uncertainty are expected to increase with the extent of phylogenetic differences between the experimental model and humans and with the relevance of the test system to human exposure, pharmacokinetics, and metabolism. Identified uncertainty factors would be combined and result in the use of a cumulative factor to determine the margin of exposure.

Addendum

After completion of this manuscript, three important publications from the Health and Environmental Sciences Institute project on Risk Assessment in the 21st Century (RISK21 Project) have appeared [99–101]. These publications are highly relevant, and are complementary to the two reports of the IWGT Working Group on Quantitative Approaches to Genetic Toxicology Risk Assessment. We call attention particularly to the discussion of the "key events/dose response framework" (KEDRF) presented in the Simon et al. [101] publication.

Conflicts of interest

J.T.M. consults for regulated industries, government agencies, and laboratories that develop and/or perform regulatory genetic toxicology tests. No other conflicts of interest were noted.

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Authors' contributions

All authors participated in the discussions that led to the consensus conclusions and recommendations reported herein, and all authors contributed to the writing and editing of this report. JTM served as Chair of the QWG and played a major role in organizing and leading the group discussions, as well as drafting and editing significant sections of this publication. The roles of Co-Chair and Rapporteur of the QWG were initially held by VT and MJS, respectively, but due to their inability to attend the meetings at Foz do Iguaçu, RF and PAW, respectively, assumed these roles. Major sections of the manuscript were initially drafted by MG and RF (extrapolation from *in vivo* and *in vitro* data), by DDL and LH-S (cancer-mutation correlations), and JTM, PAW, RF, VT (introduction, objectives, conclusions). BMD calculations were performed by LH-S and GEJ contributed to the data analysis and development of figures. DAE identified the examples in Table 1. SF provided the key data upon which Appendix 1 is based and contributed to the writing and editing of this section. LM prepared the initial draft of Appendix 2. Many QWG members participated in small sub-groups that formulated issues to be addressed and developed proposed consensus points and recommendations within sub-topic areas; leaders of these groups included JTM, RF, PAW, VT (objectives and topic identification, conclusions and recommendations), MG, MJS, TK, RF (extrapolation across systems and to humans), RF, SF (approaches to define exposure-related risk), DDL, SF, LH-S, GEJ (cancer-mutation correlations). DDL and TM managed the reference formatting and citations.

Acknowledgments

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Appendix 1. Analysis of the relationships among PoDs for carcinogenic and genotoxic effects associated with MeIQx exposure

Because cancer has been shown to be a multistep process that consists of a series of progressive key genetic, epigenetic, and cellular events that lead to uncontrolled cell proliferation [102,103], and because only a minority of early events progress to cancer, it is expected that the measurement of key events that occur earlier in this process would occur at a higher frequency and be more sensitive than later events (*i.e.*, would have lower BMD values).

One example for which good data were available is the data showing the response of key events in the liver of F344 rats exposed to the model genotoxic hepatocarcinogen 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx)[104]. Rats exposed to this carcinogen were examined to determine the formation of 1) MeIQx-DNA adducts [105], 2) mutations at the *H-ras* locus [106] and in *LacI* transgenes [107], 3) pre-neoplastic lesions (PNL) in the form of GST-positive foci [105], and 4) hepatocellular adenomas and carcinomas [108]. These endpoints were evaluated at 4, 16, 16–32 and 56 weeks of repeat dosing, respectively. In preparation for the IWGT 2013 workshop the BMD and BMDL were calculated using PROAST modeling software [109] to provide standardized metrics for each parameter for comparison. It was shown that the BMD_{10} and $BMDL_{10}$ ranking along the path of carcinogenesis was as expected; *i.e.*, earlier key events increased at earlier times and at lower doses than those closer to the apical endpoint of cancer induction (Fig. 2). The quantitative order of significant induction of the events measured was: DNA $adducts_{4weeks} \ll Mutations_{16weeks} < GST\text{-}positive\ Foci_{16–32weeks} \ll Cancer_{56weeks}$.

It should be noted that the BMD_{10} for cancer induction and the BMD_{10} for the genotoxicity endpoints are not equivalent metrics. The BMD_{10} for cancer induction is based on an absolute 10% increase in the incidence of tumor-bearing animals whereas the BMD_{10} for genotoxicity induction is based on an increase equal to 10% of the spontaneous damage rate in the group of animals examined (see [2]). Thus, the selected metrics for genotoxicity damage are inherently much more conservative, in that they reflect the sensitivity of the genotoxicity assays to detect much smaller increases relative to the existing background rate than does the cancer bioassay.

Because tumor sequencing studies suggest that over time the cells which become tumor cells may accumulate multiple "driver" mutations among an even larger number of "passenger" mutations that do not confer selective growth advantage [110], a mutation can be one of several "key" (*i.e.* necessary) events in tumor induction and so knowledge of the rate-limiting key steps in mutagenesis and carcinogenesis is important when attempting quantitative predictions. Correlative data such as that above is necessary to achieve this understanding.

It is unfortunate that other examples with similar extensive data sets could not be identified by the QWG, and it is recommended that such comparable data should be developed for other carcinogenic agents to allow further evaluation of the extent to which quantitative predictions about cancer outcome can be made based on quantitative analysis of mutagenic events in the carcinogenesis process. Because studies from different laboratories are often conducted for different purposes, when attempting to combine studies from different literature reports involving the endpoints from a pathway of interest, it is rare that studies of a compound conducted by different labs all use the same experimental conditions and route of exposure, species, strain, sex, and target tissue of the animals under study. Thus, we suggest that it may be necessary to prospectively undertake studies designed to investigate the sensitivity of key genetic events relative to cancer outcome in order to provide the information necessary to bring such analyses into practical regulatory use. Options for obtaining such data could be the inclusion of genetic measurements in animals already undergoing carcinogenesis studies or in satellite groups of animals with appropriate transgenic markers.

Appendix 2. EMS/Viracept® case study: example of a successful quantitative risk assessment

The need for a quantitative risk assessment of human exposure to ethyl methanesulfonate (EMS) was triggered by an accidental exposure of patients to EMS after ingesting contaminated Viracept® (Nelfinavir mesylate) tablets. In early 2007, it was discovered that the impurity had formed over a period of time in a storage tank for methanesulfonic acid after the cleaning fluid (ethanol) was not properly removed prior to refilling. The contaminant remained during the synthesis process and was found in the finished product at levels reaching approximately 1000 ppm. Retrospective analysis

Table 2
Analysis of NOEL and breakpoint dose for EMS [111,112].

Study	Organ	NOEL (mg/kg)	Breakpoint dose (BPD) (mg/kg)	95% Confidence interval of BPD (mg/kg)
MNT	Bone marrow	80	89.8	56.7–118.2
Muta™mouse	Bone marrow	25	35.4	21.5–45.7

showed that tablets produced prior to the incident had contained EMS at levels several orders of magnitude lower, verifying that Viracept® manufactured during a three month period was contaminated. However, a significant number of patients were exposed during this period. Details on this case and how it was managed can be found in the Special Issue No. 190 of Toxicology Letters [47].

In the course of the animal studies on the genotoxic activity of EMS, the clastogenic effects were investigated in bone marrow of mice and the mutagenic effects were studied in bone marrow, liver and large intestine. Further, free EMS was determined in the circulation and globin adducts in blood were determined in both studies. Complete dose–response data were obtained for the gene mutation study. As the studies were designed to determine a no-effect level of exposure and to investigate the hypothesis that there was a threshold for mutation induction, it was of utmost importance to test the effect of various dose levels below the putative threshold for mutations. When analyzing the data on micronucleus induction in the bone marrow of CD1 mice, *lacZ* mutation induction in the bone marrow of Muta™Mouse mice, and, for both studies, the induction of globin adducts in peripheral blood, it became apparent that low-dose levels do not result in increases in these genotoxic effects but did substantially increase the adduct levels. Above the NOEL doses of 25 mg/kg/day for *lacZ* induction and 80 mg/kg/day for MN induction, clear increases of the genotoxic effects were observed, reaching a factor of 8.7- (MN) or 4.0-fold (*lacZ*) above control values. Using the bilinear modeling software developed by Lutz and Lutz [112], estimates of the breakpoint below which the slope of the dose response curve did not differ from zero and confidence intervals were determined and are shown in Table 2.

Regarding adduct levels in globin, it was evident that no threshold for adduct formation could be derived from the data. In fact, an almost 10-fold increase over background was apparent already at the lowest dose of about 1 mg/kg/day. At the no-effect doses for genotoxicity, the ethylvaline levels surpassed the background values by roughly a factor of 1000. In liver cells of animals treated at the NOEL dose for *lacZ* mutation induction (50 mg/kg/day), it was calculated that each daily EMS dose induced 380,000 DNA alkylations without any measurable increase in mutation frequency. For bone marrow cells, the calculation yielded a total of 78,000 adducts at the NOEL of 25 mg/kg/day, assuming a similar adduct induction in bone marrow DNA as in liver DNA. The difference in calculated adduct levels between the two organs might be due to the different cell turnover (*i.e.*, the liver cells have more time to repair adducts before replication).

These data, together with information on the type of DNA adducts induced by EMS and their removal, supported the hypothesis of a threshold mechanism for mutation induction of EMS with a level below which DNA repair was assumed to prevent mutation induction. Ethylation by EMS (and methylation by MMS) occurs predominantly at nitrogen sites (N^7-G in DNA, cysteine nitrogens and terminal nitrogens in proteins), while oxygen sites within DNA are targeted to a larger extent by ENU/MNU (O^6-G, O^2-T). For removal of the different DNA lesions, various repair mechanisms are available.

These analyses were presented to the responsible regulatory authority (EMA), which concurred that the data and analyses established that the exposures experienced presented negligible risk to the exposed patients. Therefore no cancer registry was required to be established and health follow-ups were not required. Thus, this case demonstrates that a quantitative risk analysis with good experimental data combined with mechanistic and exposure information can allow the conclusion of negligible risk at a calculated margin below the derived PoDs, and that the concurrence of the responsible regulatory authorities can be successfully achieved. This example also illustrates the value of good exposure and PK information combined with a mechanistic understanding of cellular protective mechanisms that allows uncertainty factors to be minimized.

More recently, Cao et al. [113] have conducted experiments in which male *gpt*-delta transgenic mice were treated daily for 28 days with 5–100 mg/kg EMS, and measurements were made on: (i) *gpt* mutant frequencies in liver, lung, bone marrow, kidney, small intestine, and spleen; and (ii) *Pig-a* mutant frequencies in peripheral blood reticulocytes (RETs) and total red blood cells. MN induction also was measured in peripheral blood RETs. These data were used to calculate Points of Departure (PoDs) for the dose responses, *i.e.*, no-observed-genotoxic-effect-levels (NOGELs), lower confidence limits of threshold effect levels (Td-LCIs), and lower confidence limits of 10% benchmark response rates ($BMDL_{10}$). Similar PoDs were calculated from the published EMS dose–responses for *LacZ* mutation and CD1 MN induction. Vehicle control *gpt* and *Pig-a* MFs were 13–40-fold lower than published vehicle control *LacZ* MFs. In general, the EMS genotoxicity dose–responses in *gpt*-delta mice had lower PoDs than those calculated from the Muta™Mouse and CD1 mouse data.

The results indicated that the magnitude and possibly the shape of mutagenicity dose responses can differ among *in vivo* models, with lower PoDs generally detected by gene mutation assays with lower backgrounds. Thus, the data published by Cao indicate a lower observed effect level in *gpt*-delta mice than observed in *lacZ* mice. While the *lacZ* data indicate a NOGEL of approximately 25 mg/kg/day, the NOGELs for the *gpt*-delta model seem to lie below 13 mg/kg/day with a lowest effect level found in the lung at 5 mg/kg/day. Thus, while the damage-specific DNA repair pathways for EMS support in principle a sub-linear dose–response for mutations induced by EMS, it appears to be advantageous to study mutations in a model with a low spontaneous background *versus* a higher background when determining low dose risk and NOGELs.

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