Table 3
Results from Japanese CSCL and ISHL databases of *in vitro* genotoxicity tests for chemicals tested either for carcinogenicity or *in vivo* genotoxicity (including chemicals overlapping with other databases).

		No. of chemicals showing *in vitro* profile of results/total			
In vitro results for Ames-positive chemicals		Carcinogenicity results		*In vivo* genotoxicity results (not tested for carcinogenicity)	
CAvit or MNvit	Gene mutation (*Tk*$^{+/-}$ or *Hprt*)	Carc	Non-carc	In vivo positive (IVP)	In vivo negative (IVN)
+	+	19/23	3/5	1/1	0/0
+	–	0/23	1/5	0/1	0/0
–	+	3/23	0/5	0/1	0/0
–	–	1/23	1/5	0/1	0/0
+	ND	15/15	5/6	3/4	7/10
–	ND	0/15	1/6	1/4	3/10

+ = positive; - = negative; ND = not determined.

two mammalian cell tests were available for 10 out of the 11 Ames-positive substances: a test for gene mutation and either a test for clastogenicity (CAvit) or the *in vitro* MN test (MNvit). Long-term toxicity studies in rats and mice were available for all substances. Mechanistic data were available for some pesticide active substances in order to demonstrate a non-genotoxic mode of action for carcinogenicity. However, these data were not collected and were not taken into account in the analysis.

Results were categorised as positive, negative or equivocal on a weight of evidence approach since it might be that more than one study was available for some endpoints. Results are detailed in Table 4.

The 7 out of 10 Ames-positive substances that were found positive in at least one mammalian cell test were either rodent carcinogens or genotoxic *in vivo*. By contrast, the 3 Ames-positive substances that were negative in the mammalian cell tests were neither rodent carcinogens nor *in vivo* genotoxic compounds. A further analysis of the Ames test results for these 3 substances is described below:

- Dinocap: the positive results in the Ames test were observed with dinocap technical of low purity. Purified samples of dinocap were not mutagenic in the Ames test. Therefore, positive results in the Ames test might be due to the presence of genotoxic impurities.
- Ethephon: the test substance was only positive in TA 1535 with and without S9.
- Fenitrothion: mutagenicity was observed only in TA 100 and it was enhanced with metabolic activation. Mutagenicity was not observed in a nitroreductase-deficient strain, TA100 NR, and it decreased in a transacetylase-deficient strain, TA100/1,8-DNP6. The results suggest that bacterial nitroreductase activity is necessary for fenitrothion to express mutagenicity.

2.5. Analysis of data from SCCS

V. Rogiers presented data that had been collected at the Vrije Universiteit Brussel (VUB) by herself, Tatyana Y. Doktorova and Gamze Ates, and has been published [49]. For this survey, the VUB's Cosmetics ingredients DataBase (CosDB) was used, which contains toxicological information on cosmetic ingredients that are present in the Annexes of the Cosmetic Directive 76/768/EEC (preservatives, UV-filters, colorants and specially studied substances with a potential risk for human health such as hair dyes). The data was extracted from the opinions generated in the period between 2000 and 2012 by the Scientific Committee on Cosmetic Products and non-food products (SCC(NF)P) and Scientific Committee on Consumer Safety (SCCS) [50]. For the purpose of the workshop, only the genotoxicity test results were evaluated, and in particular the substances showing a positive Ames test were selected.

The survey provided 52 Ames-positive compounds with quality data (evaluated by the experts of the SCC(NF)P and SCCS). Analysis of data from the other *in vitro* genotoxicity assays performed on the Ames-positive compounds showed that the mammalian cell gene mutation test on *Tk*$^{+/-}$ or *Hprt* loci (49/52) and the CAvit (38/52) are the most commonly performed *in vitro* tests. Interestingly, 20 (including equivocal results) out of the 49 mammalian cell gene mutation assays were also positive, whereas for the CAvit, 25 (including equivocal results) out of the 38 compounds also tested positive. A survey of the outcome of the follow-up *in vivo* tests carried out for those compounds showed that a positive Ames test resulted in only 2 compounds that were genotoxic *in vivo* (4% Ames-positive compounds confirmed *in vivo*). The other 50 compounds produced negative results in the follow-up *in vivo* genotoxicity tests (96% "false" positives based on the Ames test). Only 8 out of the 52 scrutinised compounds had carcinogenicity data (2-year rodent carcinogenicity assay).

A more detailed examination of the data showed that 47 out of the 52 Ames-positive compounds were identified as hair dyes (19 semi-permanent hair dyes, 16 oxidative hair dyes and 12 hair dyes that can be used either as a semi-permanent hair dye or as an oxidative hair dye depending on the presence of peroxide). Analysis of the number of "false" positives was performed for each of the 3 separate groups, but the outcome did not differ significantly from the general analysis of the 52 compounds.

Finally, the hypothesis as to whether Ames-positive results could be overruled by 2 negative *in vitro* mammalian cell assays was challenged. It appeared that 9 out of the 52 Ames-positive compounds were associated with 2 negative *in vitro* mammalian cell tests. The concomitant *in vivo* genotoxicity test results were negative as such classifying the Ames test results as being "false" positive. In 7 out of the 9 cases the positive results were obtained in *Salmonella typhimurium* TA98 strain in the presence of S9.

2.6. Review of ECHA database

At the time of the workshop, data on industrial chemicals submitted to the European Chemicals Agency (ECHA) under the REACH legislation were not available. However, lists of chemicals for which carcinogenicity *in vivo*, and *in vitro* genotoxicity data were retrieved from the ECHA's registration database by P. Karamertzanis and F. Le Curieux, and were subsequently analysed by Ph. Vanparys on behalf of EURL ECVAM.

The dossiers considered for the current evaluation were retrieved from ECHA's registration database (http://echa.europa.eu/information-on-chemicals/registered-substances; last accessed 22 July 2014) by applying a set of filters. The first filter that was applied was to select lead and individual dossiers that fulfilled the information requirements of REACH Annex IX or X and were submitted prior to the 2010 registration deadline. This filter produced a set of 1904 dossiers that were subsequently analysed with regard to the genotoxicity, carcinogenicity and classification information they contained. A second filter selected dossiers with a positive

Table 4
Results from Ames-positive pesticides from the European Pesticide Peer Review Database in mammalian cell tests, *in vivo* genotoxicity and carcinogenicity studies.

Pesticide	CAS No.	Gene mutation (*Hprt* or *Tk*$^{+/-}$)	MNvit or CAvit	Some evidence of *in vivo* genotoxicity[a]	Rodent carcinogenicity
Carbendazim	10605-21-7	–	+	+	+
Dichlorvos	62-73-7	+	+	+	E
Dinocap	39300-45-3	–	–	–	–
Ethephon	16672-87-0	–	–	–	–
Fenitrothion	122-14-5	–	–	–	–
Parathion-methyl	298-00-0	+	+	+	–
Phosmet	732-11-6	+	–	–	+
Tri-allate	2303-17-5	–	n.a	–	+
Acetochlor	34256-82-1	+	+	+	+
1,3-dichloropropene	542-75-6	–	+	+	+
2-phenylphenol	90-43-7	+	+	E	+

+ = Positive; – = negative; E = equivocal; n.a. = not available.
[a] For the purposes of this analysis the criterion applied to define an "*in vivo* genotoxic compound" was when there was some indication of genotoxicity *in vivo*.

Ames study. The positive result had to be in an endpoint study record that according to the registrant's assessment was considered reliable (reliability 1 or 2). A reliability score of 1 means that the study is reliable without restriction, while a reliability score 2 is related to studies for which there are some restrictions (*e.g.* no reference to OECD Test Guidelines, deviation from OECD Test Guidelines, significant methodological deficiencies, *etc.*). The algorithm did not attempt to recognise weight of evidence in Ames, *i.e.* one reliable positive result was sufficient to select the dossier, regardless of the outcome of other Ames studies. At this stage, study results based on read-across were also included. This approach led to approximately 400 dossiers with evidence of positive Ames results that were then used to contrast the positive Ames outcome with *in vivo* mutagenicity and carcinogenicity data in the dossier. Because identifying cancer evidence in carcinogenicity studies is not straightforward, the algorithm used the carcinogenicity classification. In some cases a harmonised classification for carcinogenicity was available, (*i.e.* Globally Harmonized System of Classification and Labeling of Chemicals (GHS) categories C1A, C1B or C2), in others the substances had been self-classified by the registrants [51]. The dossiers with evidence of positive Ames results were divided into the following four lists:

- Ames positive chemicals that were negative for carcinogenicity (list 1): at least one reliable carcinogenicity study AND no harmonised and self-classification as C1A, C 1B or C2 [51]. List 1 contained 72 dossiers related to 71 unique substances.
- Ames positive chemicals that were negative for *in vivo* genotoxicity (list 2): at least one reliable *in vivo* genotoxicity study with no positive results, and no harmonised or self-classification as germ cell mutagen M1A, M1B or M2 (as also defined in [51]). List 2 contained 154 dossiers related to 154 unique substances.
- Ames positive chemicals that were positive for carcinogenicity (list 3): the availability of a harmonised or a self-classification as carcinogen C1A, C1B or C2 [51]. List 3 contained 280 dossiers related to 266 unique substances.
- Ames positive chemicals that were positive for *in vivo* genotoxicity (list 4): at least one reliable *in vivo* genotoxicity study, and at least one positive result in a reliable *in vivo* genotoxicity, or the availability of a harmonised or a self-classification as germ cell mutagen M1A, M1B or M2 [51]. List 4 contained 240 dossiers related to 226 unique substances.

There were dossiers that could be found in more than one of these lists. After this automated procedure the lists were manually examined and post-processed. In particular, the following substances were removed: petroleum derivatives (which accounted for the majority of the substances), substances duplicated within the other databases considered for the overall analysis (9 chemicals overlapped with NTP, 11 with the Japanese CSCL Database, 11 with CGX [9], 1 with DG SANCO SSCS and Cosmetics Industry DB), polymers and inorganic compounds with no structure and/or no molecular weight, and mixtures. This resulted in a final list of 73 registered dossiers corresponding to 73 unique substances.

These 73 selected dossiers were downloaded from the ECHA dissemination website and reviewed. At this stage, read-across data were not considered. Tests that were reviewed for the purpose of this analysis were: Ames test, CAvit, MNvit, MLA, *Hprt*, CAviv, MNviv, UDSviv, *in vivo* comet, and transgenic mouse models. For carcinogenicity the study reports were reviewed and the classification (harmonized or self-) was reported when available. A positive, negative, inconclusive or equivocal call was given for each test result. Since for some chemicals more than one study per test was available, a total of 1062 studies were reviewed. Among these studies some were considered not informative for the purpose of this exercise (*e.g.* read-across). Subsequently, for each test method an overall call was given following the same criteria as those used in the construction of a consolidated database (see [52]). The review of the ECHA dossiers relied entirely on the information submitted by the applicant.

In several cases the information reported was not sufficient to allow a firm overall conclusion for a certain test in accordance with the criteria defined. This was the case with negative or unclear results, where no firm conclusion could be made in terms of requirements specified in current OECD Test Guidelines or recommended best practices (*e.g.* studies only without S9 or only with S9, abnormal pH levels or osmolality, not the standard set of bacterial strains, no adequate levels of toxicity, no proof of target cell exposure *in vivo*, *etc.*). Often the submitted studies reported only the conclusions and omitted the original data; in other cases, references were made to reports which were not accessible or where references were not available. Thus, for the above reasons, despite the massive review work, several studies were given an overall "inconclusive" call and were as such not considered informative.

The re-evaluation of the 73 compounds resulted in 29 Ames-positive chemicals, 12 Ames-equivocal, 16 Ames-inconclusive, and 16 Ames-negative. These data were considered insufficient to allow a meaningful analysis of the ECHA results in isolation. However, it was decided to add these data to a new consolidated database [52], but it was necessary to conduct a further search of literature data with the aim of clarifying some of the inconclusive or equivocal calls. ECHA data, which resulted from the subsequent expert and literature review, and added to a consolidated database [52] therefore represent data from 32 chemicals that were submitted to ECHA for the 2010 registration deadline.

2.7. Review of industry data

2.7.1. BASF data on pesticides and industrial chemicals

M. Schulz (BASF, Germany) reported that data on positive Ames studies and *in vivo* follow-ups is rare for pesticides, as a positive Ames test result is in general a cut-off criterion for the development of new chemical entities. Nevertheless, the occurrence of positive findings in bacterial reverse mutation assays with industrial chemicals and pesticides at BASF was extremely low over the last 7 years with only 19 out of 574 compounds (3.3%) giving positive Ames results. It should be noted that from this set of 19 positives, 6 test materials were complex mixtures. Twelve of these 574 substances were tested in the rodent bioassay and failed to induce tumours, whereas 2 of these gave positive results either in the Ames test or in an additional mammalian cell genotoxicity test (*Hprt* mutation or CAvit). Both substances were biocides, so the *in vitro* observations were not that surprising.

2.7.2. Pharmaceutical industry

A. Lynch (GSK, UK) reported that the Ames Test for mutagenicity is a strong predictor of genotoxic carcinogenicity and is a pivotal 'must do' in progressing a drug for non-life threatening indications, where positive results often represent a "no go" decision for further development.

Since 2009, GSK has tested 976 compounds in the Ames test; these have included active pharmaceutical ingredients (APIs) and drug synthesis intermediates, impurities, degradants and, on occasion, human-specific metabolites. Of these, the majority of API's were negative (86.3%). Of the 134 positive API's (13.7%), 37 compounds were positive only in the presence of induced rat liver S9 metabolic activation (27.6% of positives, 3.8% of compounds tested). The development of an Ames-positive API is generally terminated early in the absence of a positive risk/benefit assessment. Such compounds are seldom assessed *in vivo* for genotoxicity or carcinogenicity, and there are only 11 examples of APIs in the GSK database with positive Ames results and additional *in vivo* genotoxicity and/or carcinogenicity data (Table 5). One reason for this is the positive impact of quantitative structural activity relationship (QSAR) assessment on drug design where careful consideration of chemical structure during lead-drug optimisation avoids the synthesis of API with overt mutagenic structures.

Reactive chemistry is still the mainstay of small molecule drug synthesis and the generation of the final API can often require several intermediate steps. The control of drug synthesis intermediates and impurities in the final API is therefore an important consideration for worker safety and for patients, respectively. Where possible, manufacturing procedures are put in place to eliminate and/or control levels of contamination. Ames-positive drug synthesis intermediates and impurities are generally controlled to the threshold of toxicological concern (TTC) and these compounds are also rarely assessed for *in vivo* genotoxicity or carcinogenicity. The methods on which the TTC value is based are generally considered to be extremely conservative as they involve a simple linear extrapolation from the dose giving a 50% tumour incidence (TD_{50}) to a 1 in 10^{-6} incidence using TD_{50} data for the most sensitive species and most sensitive site *i.e.* several worst case assumptions [53]. As indicated in the European Medicines Agency (EMA) guideline on limits of genotoxic impurities in pharmaceutical products [54], a TTC value of 1.5 μg/day is expected to result in no more than a 10^{-5} lifetime cancer risk. Compounds which cannot be controlled to the TTC either require extensive follow-up testing with additional *in vivo* (geno)toxicology studies to determine their potential carcinogenic risk, or alternative synthetic routes can be considered which eliminates the compound of concern. There are 19 examples of intermediates/impurities in the GSK database with Ames-positive results and additional *in vivo* genotoxicity and/or carcinogenicity data (Table 5).

Ames-positive degradation products and/or human specific metabolites represent another category of compounds which present a significant challenge. These compounds are often only identified later on during drug development. One such degradant is contained in the GSK database of Ames-positive compounds (Table 5).

2.7.3. Cosmetics industry

Genotoxicity data that were generated in conjunction with the submission of safety dossiers for hair dye ingredients as mandated by the European Commission (currently SCCS) were presented by S. Pfuhler (The Procter & Gamble Company, Cincinnati, OH). The majority of the data that went into the industry dossier submissions was generated between 2003 and 2009, and those studies were performed in compliance with OECD Test Guidelines and GLP. The dossiers were reviewed by the SSCS and the outcomes (the so-called opinions) are published on the respective website of the European Commission [50]. Thus, most of the data here presented are related to substances also reviewed in Section 2.5.

An overview of all genotoxicity data for those dyes that have shown unfavourable (*i.e.* positive) Ames results ($n=27$) was presented to the workshop. For all dyes, data from one to three mammalian cell-based *in vitro* studies was available, as well as data from at least one *in vivo* follow-up study (mostly two). All *in vivo* follow-up studies were negative with one exception that will be discussed later, thereby supporting the assumption that the positive results observed in the Ames test may not be relevant for the safety assessment of these compounds. The profile of the *in vitro* mammalian cell results, on the other hand, was very inconsistent. The mammalian cell data do not seem to give a clear picture in terms of their value in predicting the *in vivo* genotoxicity potential of these dyes, however there were several dyes that have a signature that seems worth mentioning. Five out of 27 Ames-positive hair dyes exhibited a negative profile in the mammalian cell-based studies performed. In these cases the *in vitro* mammalian cell data agreed with the negative *in vivo* data, which supports the value of such a data constellation for predicting the absence of a genotoxic potential *in vivo*.

Table 5
Summary of Ames-positive compounds from the GSK database with *in vivo* genotoxicity and/or carcinogenicity data.

Tot No.	DEREK alerts	Positive BlueScreen-GreenScreen (total)	Positive Ames (total)		*In vitro* mammalian positive (total)			Positive *in vivo*			Carc (Total)	Comments
			+S9	-S9	MLA (*Tk*$^{+/-}$)	CA	Other	MN	Comet	UDS		
38	23	12 (13)	28	29	14 (22)	6 (8)	2 (4)	0 (38)	0 (5)	1 (6)	3 (6)	11 API 19 interm. 1 degradant

Interm. = intermediate; API = active pharmaceutical ingredient.

Table 6
Factors affecting chemical accessibility, metabolism and toxicity in bacteria, mammalian cells and intact mammals.

Bacteria	Mammalian cells	Mammals
Circular DNA (no nucleus)	Nuclear chromosomes with associated proteins and histones	Nuclear chromosomes with associated proteins and histones
Cell wall	Plasma membrane	Plasma membrane
Single cell, exposure *via* immediate environment	Single cell or monolayers, exposure *via* solution	Tissue and organs, exposure *via* ADME (bloodstream)
Limited xenobiotic metabolism	Limited xenobiotic metabolism	Extensive xenobiotic metabolism
Limited antioxidant activity	Some antioxidant activity	Full antioxidant activity
Response to stress/toxicity is mutation	Response to stress/toxicity is cell death	Response to stress/toxicity is cell death
Default to a toxic environment is survival through mutation	Default to a toxic environment is cell death	Default to a toxic exposure is cell death
Response to toxicity is dose dependent	Response to toxicity is dose-dependent with a low threshold	Response to toxicity is dose-dependent with highest possible threshold
DNA damage repair is geared to survival/mutation	DNA damage repair is geared to fidelity	DNA damage repair is geared to fidelity

By contrast, the only dye that exhibited positive results *in vivo* (1-hydroxy-2-amino-5-metyhylbenzene; mouse micronucleus assay) has a very different *in vitro* profile, and gave positive effects in all mammalian cell assays that were performed. While the *in vivo* positive result, observed in a study using oral gavage, is pointing towards a genotoxic hazard it is important to mention that this dye will be activated by phase I hydroxylation but detoxified by N-acetylation, a phase II reaction prevalent in the skin [55–57]. The fact that this dye produced an increase in the frequency of bone marrow micronuclei after oral exposure and was positive in the mammalian cell assays, however, is supportive of the use of an *in vitro* only follow-up strategy for Ames-positive compounds, in that two mammalian cell studies with favourable outcomes can be taken as an indication of absence of *in vivo* genotoxic potential.

2.8. Metabolic considerations

N. Gooderham presented his views on "The role of metabolism in genotoxicity – the dynamic dimension". The requirement for metabolism in mammals has almost certainly evolved through competition between plants and animals for survival. As part of a defence mechanism to dissuade mobile competitors (insects and higher order species) from consuming them, plants synthesise chemicals that are distasteful or harmful to higher order organisms. In many cases these chemicals have no nutritional value and can be inherently toxic. Thus during evolution, mammals developed strategies to eliminate or reduce their exposure to these foreign chemicals (xenobiotics) when consumed as part of their diet. Such compounds are generally lipophilic and once they have access to the body, they would accumulate in fatty tissue. Mammalian metabolism has evolved to convert these lipophilic materials to polar derivatives to facilitate their excretion and elimination from the organism. The chemical diversity of these xenobiotics is enormous, thus the enzyme systems that have evolved to deal with this array of structures have broad substrate specificity at the expense of the high catalytic efficiency normally associated with biochemistry. In the higher order species such as mammals, the diversity of structure that metabolism can process is truly remarkable. These metabolic systems transform xenobiotics, dependent upon the chemical's physiochemical characteristics (*e.g.* lipophilicity, pKa), using and manipulating functional groups with the objective of bestowing polarity on the molecule [58]. In understanding the mammal's ability to achieve this objective, we can define discrete processes involved as the chemical journeys through the body, specifically absorption, distribution, metabolism and elimination (ADME).

Each of these steps involves interaction of the xenobiotic with biological biomolecules and the entire process is highly dynamic. There are competing pathways and thus multiple metabolic products (metabolites), kinetic considerations, functionalisation and conjugation reactions, competing chemistry, and enzyme inhibition/induction effects [59]. All of these elements can alter the metabolic fate, and thus the toxicity, of xenobiotics. Other considerations include qualitative issues such as pathway deficiency (*e.g.* sulphation in the pig), enzyme polymorphism, precursor deficiency (*e.g.* sulphate levels), and tissue and species specificity [60].

Metabolism is usually geared to detoxication as the compound is rendered polar and thus excretable, but sometimes metabolism results in a more damaging product being generated, leading to toxicity. Detoxication and activation processes occur within the same temporal frame and at any specific time the balance between activation and deactivation determines toxicity and hence a toxicity threshold.

Since mammalian metabolism capacity is the product of evolution, there are significantly different responses to xenobiotics in bacteria compared to mammals. Specifically, there can be species specific metabolism, differing detoxication thresholds, differing oxidative damage responses, chemical reactivity of the xenobiotic with a species-specific biomolecule resulting in the formation of a reactive product, and species-specific responses to toxicity (see Table 6).

Thus, bacteria and mammals have very different metabolism capability and this ability to activate or deactivate xenobiotics has led to the evolution of different outcome strategies to address toxicity (Table 6). The bacterial response to toxicity is mutation to adapt and survive, whereas the mammalian cell default to toxicity is cell death (apoptosis/necrosis). Examples of metabolic differences between bacteria and mammalian cells include bacterial alkyl hydroperoxide reductase, used as a defence against DNA oxidative damage [61]; high levels of reductive enzymes, compared to mammalian cells, permitting bacteria to efficiently activate nitro and azo compounds to electrophilic metabolites [62]; limited phase 2 conjugation capacity, although bacteria are usually proficient in glutathione S-transferase activity [63]. In current *S. typhimurium* bacterial mutagenicity assays, to circumvent this limited metabolism capacity, the incubation of bacteria with xenobiotics is supplemented by adding mammalian hepatic S9, a partially purified tissue preparation rich in enzymes involved in xenobiotic metabolism. Traditionally this S9 is prepared from the livers of rats treated with mixtures of chemicals known to induce hepatic drug metabolism. Typically the inducer will be Aroclor 1254 (a mixture of polychlorinated biphenyls) or a phenobarbital/β-naphthoflavone mixture. Both treatments preferentially induce oxidative liver enzymes, particularly cytochromes P450. Inclusion of induced rat liver S9 in bacterial mutagenicity assays generates a highly oxidising catalytic environment that favours oxidative

activation and bears little resemblance to human metabolic capacity. The induced S9 preparation has limited phase 2 (conjugation metabolism) activity due to deficient concentrations of obligatory co-factors. It should also be noted that the metabolising potential of S9 is species specific.

Intact mammalian cells have an active phase 1 and phase 2 metabolism capacity. Hepatocytes would express the best complement of enzymes, but are technically difficult to isolate and use. Furthermore, since primary hepatocytes are not in a proliferative phase, there is a small window of opportunity (isolation to 4–6 h) after which the metabolic capacity is compromised. Using established cell lines is a potential alternative, but these invariably are enzyme deficient, especially in regards to the range of cytochromes P450, or if engineered, express an unbalanced enzyme profile.

These fundamental metabolism differences between bacteria, mammalian cell lines and intact animals are illustrated by responses to the food-derived heterocyclic amine 2-amino-3,8-dimethylimidazo[4,5-b]quinoxaline (MeIQx). MeIQx is carcinogenic in lifetime bioassays at high doses (400–600 ppm in the diet) in both rats and mice [64,65]. In *S. typhimurium* bacterial mutation assays, MeIQx is negative in the absence of S9, but is a remarkably potent mutagen in the presence of Aroclor-induced rat liver S9 and human liver S9 [66]. In mammalian cell genotoxicity assays, MeIQx generally gives a weak response (UDS in rat and hamster hepatocytes; *Hprt* locus in CHO cells; *Hprt* locus in V79 cells; SCE in CHO cells; micronuclei in HepG2 cells) [67,68]. In *in vivo* genotoxicity assays, the response is again generally weak requiring high doses (micronuclei and SCE in C57Bl/6 mice) or negative [69]. In other *in vivo* genetic damage assays, such as formation of GST-P positive foci in rats, the trend is repeated with positive foci only being scored at high doses [70]. In these experiments, there was evidence of a dose threshold effect for GST-P foci, yet in the same animals ^{32}P-postlabelling analysis suggested clear dose-dependency of adduct formation even at very low dose exposures [71]. This has led Wei et al. [72] to propose that a practical threshold dose exists for this genotoxic carcinogen. These dose-dependent effects can be rationalised on the basis of switching the MeIQx metabolic profile. Under the metabolic conditions used in the Ames test and in high dose *in vivo* studies, oxidative metabolism of MeIQx to its genotoxic N-hydroxy metabolite is favoured. Analysis of the kinetics of MeIQx metabolism in the rat indicates that at steady state the catalytic efficiency (K_{cat}/K_m) for the primary oxidative pathways is C5-OH > N-OH > C8-OH. At low doses of MeIQx the substrate is limiting and C5-oxidation predominates. Formation of the N-hydroxy metabolite (genotoxic) is limited by substrate availability and removal by C8-oxidation and glucuronidation. At high doses, the oxidative pathways are saturated and the N-hydroxy metabolite accumulates due to substrate availability and inefficient C8-oxidation and limiting glucuronidation.

These considerations indicate that it is possible to have an Ames positive result under conditions that favour high levels of oxidative metabolism, yet performing similar experiments in mammalian cells results in less genotoxic potential or perhaps negative results. This paradigm is continued *in vivo* unless the doses of chemical used exceed the capacity of key detoxication pathways, thereby leading to metabolic switching to toxication pathways and resulting genotoxicity. Under these circumstances genotoxicity shows a pragmatic threshold that is governed by the kinetic considerations of competing metabolic pathways.

2.9. Supplementary tests

The possible role of additional *in vitro* approaches as follow-up tests was presented by Ph. Vanparys. A core *in vitro* genotoxicity battery comprising the Ames test plus the MNvit is sufficient to detect rodent carcinogens [10]. The expectation is that in the future for chemical testing, more results will be generated with the combination of the Ames test and MNvit. For most industrial sectors, development of compounds positive in the Ames test and MNvit will be stopped before going into *in vivo* tests. But compounds positive in the Ames test and negative in the MNvit will trigger additional testing. In his opinion, compounds positive in the Ames test only in the absence of an exogenous metabolic activation system (S9 mix) should be further tested in the MLA or *Hprt* test. When found negative in the *in vitro* mammalian gene mutation assays, it is reasonable to assume that the compound has no mutagenic potential in mammalian cells. Compounds found positive in the Ames test only in the presence of S9-mix should be further tested in the Ames test with a battery of different S9-mix systems to explore species differences in the metabolising pattern. If the positive findings are related to a metabolising pattern not relevant for humans, the next step is to assess the compound in an *in vitro* mammalian gene mutation test. When negative in this assay, it is also reasonable to assume that the compound has no mutagenic potential in mammalian cells. In order to define whether mutagenic potential can lead to transformations, a cell transformation assay (CTA) preferentially on Syrian hamster embryo cells (SHE) cells can be performed.

Genotoxicity may result from a DNA-reactive or non DNA-reactive mechanism of action. Transcriptomics are very useful to define the mechanism of action and to define whether the particular mechanism can lead to genotoxicity *in vivo* and to carcinogenicity [73,74]. Transcriptomics can also be very helpful for the determination of the relevance of positive findings in the *in vitro* genotoxicity tests. It would be useful if it was possible to provide clear guidance on follow-up testing, but each data set would need to be considered on a case-by-case basis.

3. Discussion

Following the individual presentations the participants formed 2 breakout groups. The groups were asked to address the following questions, respectively:

1. Are there any general trends that can be concluded from the data regarding the "risk" predicted by different patterns of *in vitro* results (whether mammalian cell tests are positive or negative in the face of Ames-positive results)? Do different patterns of *in vitro* results signify different follow-up approaches (particularly if and when *in vivo* tests are needed)?
2. What *in vitro* tests, if any, can be eliminated without diminishing the predictivity for *in vivo* genotoxicity or cancer of the standard battery? What *in vitro* tests can be added to help with the decision-making process? In what circumstances would you select additional *in vitro* studies, and which ones?

Breakout group 1 looked at the data provided in the individual databases and made some initial analyses. They noted the following:

- The incidence of carcinogens among the Ames-positive results is ~70%, but this includes genotoxic and non-genotoxic carcinogens
- The incidence of Ames-positive non-carcinogens is ~24%
- The incidence of Ames-positive compounds with negative results in 2 mammalian cell assays is:
  - 1.2% (1/86) for *in vivo* genotoxic compounds
  - 3.3% (9/211) for carcinogens
  - 21.4% (15/70) for *in vivo* non-genotoxic compounds
  - 22.6% (12/53) for non-carcinogens.

Thus the group concluded that, on the basis of this limited analysis, if an Ames-positive compound shows negative results in

well-performed *in vitro* mammalian cell tests for both chromosomal damage and gene mutations, the compound tested is unlikely to be an *in vivo* genotoxin or a carcinogen. If supporting evidence from mechanistic studies is available then further (*in vivo*) testing should not be necessary. Thus, if a 2 test battery of Ames and MNvit is used, and gives positive results in the Ames test but negative results in the MNvit, then a second mammalian cell assay (with gene mutation endpoints) should be performed.

However, the group noted that bias could result from the small sample sizes of Ames-positive chemicals giving negative results in 2 mammalian cell tests. There could also be sample bias associated with the number of tests of a particular assay type. It was noted that there was over-representation of carcinogens and of certain chemical classes in some of the databases, and that these databases consisted of a large number of old mammalian cell studies in which the criteria for positive or negative outcomes would most likely have been based on criteria no longer used.

Breakout group 2 noted that for an Ames-positive result it is important to investigate the response pattern, *e.g.*, are the results positive in the absence or presence of S9, in which strains are the responses seen, and at which magnitude. Based on these results is it possible to generate a hypothesis why the Ames test is positive? Addressing such a question should include analysis of the chemical structure (are there structural alerts?), and try to understand if there are metabolic pathways that are unlikely to be relevant for humans (*e.g.*, nitroreduction in bacteria). Another possible explanation is that many of the azo dyes are not very pure, and the positive Ames test result for these chemicals may be produced by low levels of mutagenic impurities that do not need azo reduction for their mutagenicity. Because of the lower sensitivity of many of the mammalian cell assays, these impurities may not be present at levels sufficient for the mammalian cell response.

This group also discussed the situation where a chemical is positive in the Ames test but negative in the MNvit. In such a case, it is important to check the purity of the test material, because a trace contaminant may have a serious impact on the results.

Such results also need to be considered in light of differences in metabolism between the Ames and mammalian cell tests, for example:

- Different S9 concentrations are used, most usually 10–30% S9 mix in the Ames test, but 2–4% S9 mix in the *in vitro* mammalian cell tests.
- The duration of exposure is longer in the Ames test as compared to the mammalian cell tests.
- The intrinsic metabolism of the bacterial and mammalian cells may produce different metabolic profiles with a test chemical.
- The S9 mix used favours oxidative metabolism. Bacteria may be less able to defend against such oxidative metabolites whereas mammalian cells may have sufficient phase II metabolism to promote detoxification.

Modifications to the standard assays were also considered as being useful. Standard S9 is supplemented with co-factors for phase I activation enzymes. It may be useful to include an additional arm to a standard study that includes co-factors for phase II metabolism. To further understand whether the results using rat liver S9 are relevant, metabolic profiling could be performed in induced rat hepatocytes and compared with human hepatocytes.

With Ames-positive mammalian-cell negative chemicals it is also worth considering whether the test compound induces oxidative stress. Bacteria and mammalian cells have different capabilities to cope with specific oxidative stress. Some Ames strains are more susceptible to oxidative stress (*e.g.* TA97, TA100, TA102), and positive responses in these strains can give a hint that oxidative stress may be responsible for the positive findings. An option would therefore be to modify the Ames test design by addition of anti-oxidants, although care should be taken that this does not affect metabolic activation by S9. Another option would be to perform specific biochemical assays addressing oxidative stress.

The use of the 3D-skin models may be useful to follow-up Ames-positive results with dermally exposed chemicals. A negative comet assay in a 3D-skin model would tend to confirm the negative mammalian cell result showing that there is no primary DNA-damage. Alternatively co-culture systems (using 3D-skin and mammalian cells) can be used for this purpose as well.

CTAs can provide some additional information for weight of evidence assessments to help in decision making especially when no *in vivo* testing is allowed. Test systems, where the target is a stress gene (*e.g.*, Greenscreen/Bluescreen with GADD45) may also be useful. However, these test systems have not yet been evaluated in the context of Ames test positive responses/mammalian test negatives, as addressed in this workshop. Toxicogenomic approaches may also provide some additional information on mode of action.

The strengths and weaknesses of these different follow-up approaches are summarised in Table 7. In general, these assays can be sub-divided into assays that give mechanistic information and assays that might be necessary to confirm a conclusion or negative mammalian cell results.

Table 7
Strengths and weaknesses of different follow-up approaches to Ames-positive MNvit-negative chemicals.

Assay type	Strengths	Weaknesses
3-D skin comet	*In vivo*-like	Not validated yet.
Metabolism studies	Help explain the positive Ames result and its relevance.	Cost, time, specialised laboratories.
Toxicogenomics	Mechanistic information	Cost, time, specialised laboratories. Not validated yet.
Repair assays	Mechanistic information	Not validated yet.
Cell transformation assay	Correlates well with rat carcinogens.	The mechanisms underlying the transformations are not known.
Greenscreen/Bluescreen	Useful in screening. Detects a wide variety of DNA-damage.	Respond to non-specific DNA-damage.
In silico studies for metabolism (*e.g.* Meteor)	Alerts for activation pathways. Meteor can give all the possible metabolites. May help to define what to do next.	Meteor does not distinguish between the metabolites (specific for bacteria and specific for mammalian cells).
In silico studies for structural alerts	Specific structural alerts. May help define which tests to select.	Applicability domain limited (new structures).

4. Conclusions and recommendations

In the final plenary discussion the workshop participants agreed that, based on the individual presentations and the initial analysis of results across the different databases, there appear to be patterns of results in mammalian cell tests that are consistent across databases and indicate whether an Ames-positive chemical is likely or not likely to be carcinogenic or genotoxic *in vivo*. In particular it appears that negative results in mammalian cell tests covering clastogenic/aneugenic and gene mutation endpoints could indicate that an Ames-positive chemical is not likely to be carcinogenic or genotoxic *in vivo*. However, there were relatively few Ames-positive chemicals in the individual databases that were negative in mammalian cell tests *in vitro* and for carcinogenicity. There was consensus that because of their regulatory implications, these preliminary results were worth following up. Given that the individual databases contained overlapping chemicals with occasionally contradictory results, it was recommended that a consolidated database be constructed that would eliminate duplication, and that this be analysed. The construction and analysis of the consolidated database is reported separately [52].

It was also acknowledged that, even if the analysis of the consolidated database shows that negative mammalian cell test results could be indicative that an Ames-positive chemical is not likely to be carcinogenic or genotoxic *in vivo*, it is unlikely that a firm conclusion could be reached, or *in vivo* testing be avoided, reduced or delayed, without further data. The suggestions discussed above for follow-up studies to investigate metabolism, oxidative stress, and effects in other *in vitro* test systems should be considered.

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Conflict of interest

The authors have declared that there are no conflicts of interest.

Disclaimer

This document represents the consensus of the participants' views expressed as individual scientists and does not necessarily represent the policies and procedures of their respective institutions.

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Development of a category approach to predict the testicular toxicity of chemical substances structurally related to ethylene glycol methyl ether

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ABSTRACT

We propose a category approach to assessing the testicular toxicity of chemicals with a similar structure to ethylene glycol methyl ether (EGME). Based on toxicity information for EGME and related chemicals and accompanied by adverse outcome pathway information on the testicular toxicity of EGME, this category was defined as chemicals that are metabolized to methoxy- or ethoxyacetic acid, a substance responsible for testicular toxicity. A Japanese chemical inventory was screened using the Hazard Evaluation Support System, which we have developed to support a category approach for predicting the repeated-dose toxicity of chemical substances. Quantitative metabolic information on the related chemicals was then considered, and seventeen chemicals were finally obtained from the inventory as a shortlist for the category. Available data in the literature shows that chemicals for which information is available on the metabolic formation of EGME, ethylene glycol ethyl ether, methoxy- or ethoxyacetic acid do in fact possess testicular toxicity, suggesting that testicular toxicity is a concern, due to metabolic activation, for the remaining chemicals. Our results clearly demonstrate practical utility of AOP-based category approach for predicting repeated-dose toxicity of chemicals.

1. Introduction

Ethylene glycol methyl ether (EGME) is an important industrial chemical that is widely used in jet fuel and ink, as a plasticizer, in the manufacture of printed circuit boards, and in photographic and dyeing applications. Due to concerns about exposure to this chemical, numerous toxicological studies have been conducted (NIOSH, 1991). Several repeated-dose toxicity studies reveal that EGME produces toxicities in multiple organs associated with the hematopoietic system, immuno system and male reproductive organs (Johanson, 2000). One of the most studied organ toxicities is testicular toxicity, which is characterized by atrophy, degeneration and necrosis of the pachytene spermatocytes, and a decrease in sperm count in rats, mice and rabbits (Foster et al., 1983; Miller et al., 1983; Nagano et al., 1984; NTP, 1993). The same class of analog, ethylene glycol ethyl ether (EGEE), also shows testicular toxicity (Johnson, 2002). Comparative studies have revealed that EGME is more toxic than EGEE in rats and mice (Foster et al., 1984; NTP, 1993). Numerous studies to explore the mechanism have revealed that the testicular toxicity of EGME and EGEE is attributable to their major metabolites, methoxyacetic acid and ethoxyacetic acid, respectively (Foster et al., 1984; Moss et al., 1985). Acetates of EGME and EGEE, which are related chemicals, have also been shown to cause testicular toxicity in mice because acetates are readily hydrolyzed to EGME and EGEE (Johanson, 2000; Johnson, 2002). EGME, EGEE and their acetates are candidates for designation as Substances of Very High Concern (SVHC) in the chemical management policy of Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) in the European Union (ECHA, 2013). It is plausible to assume that testicular toxicity will result if related chemicals are chiefly metabolized to methoxy- or ethoxyacetic acid.

Since repeated-dose toxicity is one of the key items of information for hazard evaluation, chemical regulation policies are increasingly requiring that repeated-dose toxicity data be made available for marketed but as yet untested chemicals. On the other hand, reduced animal testing is desired for both economic and animal welfare reasons. The category approach thus has potential as a useful method to reduce animal testing (Schaafsma et al., 2009;

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van Leeuwen et al., 2009). Several pioneering attempts to develop a category approach have been performed for complex regulatory endpoints (Fabjan et al., 2006; Wu et al., 2010; Blackburn et al., 2011; Ball et al., 2012, 2014; ECETOC, 2012). We have recently developed a Hazard Evaluation Support System (HESS) in which the notion of a category approach is adopted based on OECD guidance on grouping chemicals into categories (Hayashi and Sakuratani, 2011; Sakuratani et al., 2013; OECD, 2014). The HESS includes databases of repeated-dose toxicity studies and metabolic maps in rats for industrial chemicals, and has a metabolism simulator. The system has a supportive function for grouping structural analogs into categories using a category profiler. HESS is compatible to OECD QSAR Toolbox. The data and category profilers of HESS are provided to the Toolbox in a period. On the other hand, HESS is unique in that detailed data can be drawn from the attached database HESS DB, which contains dose–response data of toxicity studies and more mechanistic information (Hayashi and Sakuratani, 2011; Abe et al., 2012). We have successfully tested the category approach using HESS to predict the repeated-dose toxicity of untested chemicals by combining it with the adverse outcome pathway (AOP) concept (Yamada et al., 2012, 2013). More case studies are required, however, before this approach can be applied for regulatory use.

In this study, AOP was developed for testicular toxicity of EGME. Metabolic activation was identified as a key event linked to the adverse outcome. A category was then built based on information on the active metabolite formation and the toxicity data of EGME and structural analogs from HESS. The proposed category was evaluated with data of tested analogs from toxicological literatures outside of HESS. Finally, relevant analogs were identified in the chemical inventory of the Ministry of International Trade and Industry (MITI) of Japanese Chemical Substances Control Law (CSCL), consisting of about 16,000 chemicals by using HESS metabolism simulator and taking into account related empirical metabolic information. The results clearly demonstrated the usefulness of our approach to the primary identification of chemicals with potential testicular toxicity similar to that of EGME.

2. Materials and methods

2.1. Data sets

Table 1 shows the list of data sets of chemicals used in this study. It is the merger of Tables 2 and 3, which were from HESS for forming a category and toxicological literatures for evaluating a category, respectively. The HESS software package can be downloaded free of charge from the following URL (http://www.safe.nite.go.jp/english/kasinn/qsar/hess-e.html). The current version of HESS (version 2.8) has four sub-databases of repeated-dose toxicity studies, from which the HESS Repeated-dose Toxicity Database was selected. It contains a summary of data from about 630 toxicity studies on industrial chemicals, mainly from Japanese regulatory submissions and the National Toxicology Program (NTP). All the reports are in the public domain. Given that EGME, EGEE and their acetates are listed as candidates of SVHC (ECHA, 2013), structures of ethylene glycol alkyl ethers with linear and branched chains and their acetate were manually searched for from HESS database by visual inspections. As a result, six chemicals were chosen for building a category (Table 2). Publicly-available toxicological literature was gathered for toxicity studies of EGME and related chemicals that might be converted to methoxy- or ethoxyacetic acid by examining metabolic information (Nagano et al., 1984; Cheever et al., 1989; Poon et al., 2005). A total of 15 chemicals were retrieved from the three literature reports for category evaluation (Table 3).

2.2. Development of an adverse outcome pathway and a category

Literature on EGME metabolism and various *in vitro* and *in vivo* toxicological studies were compiled for developing an AOP, which was then built for the testicular toxic effects of EGME, since this substance has been more often studied for its mechanism of toxicity. To develop the category, a data matrix was constructed and carefully evaluated in terms of the metabolism and significant pathological changes in the testis. Testicular toxic potencies were evaluated as lowest observed adverse effect level (LOAEL) for pathological changes in the testis. Finally, a category definition was described based on the structure and metabolism of the category chemicals.

2.3. Screening a chemical inventory to obtain chemical structures that form methoxy- or ethoxyacetic acid

The OECD QSAR Toolbox Ver. 3.1 (http://www.qsartoolbox.org/) contains 11 international chemical inventories, including the TSCA (Toxic Substances Control Act), the REACH ECB (European Chemicals Bureau) and HPVC (High Production Volume Chemicals) OECD, etc., of which the Japanese MITI inventory was selected for the case

Table 1
Data set chemicals, their abbreviations and CAS numbers in the present study.

Abbreviation	Chemical name	CAS no.	Data source[a]	Compound no.[b]
EGME	Ethylene glycol methyl ether	109-86-4	H, TL	**1**
EGEE	Ethylene glycol ethyl ether	110-80-5	H, TL	**2**
EGiPE	Ethylene glycol isopropyl ether	109-59-1	H	–
EGPE	Ethylene glycol propyl ether	109-86-4	TL	–
EGtBE	Ethylene glycol *tert*-butyl ether	7580-85-0	H	–
EGBE	Ethylene glycol butyl ether	111-76-2	H, TL	–
EGMEA	Ethylene glycol methyl ether acetate	110-49-6	TL	**3**
EGEEA	Ethylene glycol ethyl ether acetate	111-15-9	TL	**4**
EGDME	Ethylene glycol dimethyl ether	110-71-4	TL	**13**
DEGDME	Diethylene glycol dimethyl ether	111-96-6	TL	**14**
EG	Ethylene glycol	107-21-1	TL	–
EGA	Ethylene glycol acetate	542-59-6	TL	–
EGDA	Ethylene glycol diacetate	111-55-7	TL	–
PGMEA	Propylene glycol methyl ether acetate	108-65-6	H	–
MHE	Methyl heptyl ether	629-32-3	TL	–
EHE	Ethyl hexyl ether	5756-43-4	TL	–
BE	Butyl ether	142-96-1	TL	–
HGDE	Hexamethylene glycol dimethyl ether	13179-98-1	TL	–

[a] These chemicals were obtained from HESS (H) for category development and toxicological literature (TL) for category evaluation.
[b] The compound number is designated in Fig. 2.