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In vitro genotoxicity testing—Can the performance be enhanced?

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ABSTRACT

The assessment of genotoxicity represents an essential component of the safety assessment of all types of substances. Several *in vitro* tests are available at different stages of development and acceptance, yet they are not considered at present sufficient to fully replace animal tests needed to evaluate the safety of substances. For an overall improvement of the traditional genotoxicity testing paradigm, several recent activities have taken place. These include the improvement of existing tests, the development of novel tests, as well as, the establishment and exploration of approaches to optimise *in vitro* testing accuracy. Furthermore, useful tools, such as databases or reference chemical lists have been developed to support advances in this field.

1. Introduction

Genotoxicity assessment is an essential component of the safety assessment of all types of substances, ranging from pharmaceuticals, industrial chemicals, pesticides, biocides, food additives, cosmetics ingredients, to veterinary drugs, relevant in the context of international legislations aiming at the protection of human and animal health. In general, the assessment of genotoxic hazard to humans follows a step-wise approach, beginning with a basic battery of *in vitro* tests followed in some cases by *in vivo* testing (ECVAM, 2013).

A variety of well-established *in vitro* assays are in place and they have been used successfully to predict genotoxicity. However, they cannot at present be considered to fully replace animal tests currently used to evaluate the safety of substances. In the last decade, considerable activities have been carried out worldwide with the aim of optimising strategies for genotoxicity testing, both with respect to the basic *in vitro* testing battery and to *in vivo* follow-up tests. This reflects the fact that the science has progressed substantially and the significant experience of 40 years of regulatory toxicology testing in this area has been acquired. In addition, the need to ensure that *in vitro* tests do not generate a high number of false positive results, which trigger unnecessary *in vivo* studies, hence generating undesirable implications for animal welfare, has been recognised.

This manuscript reviews some of the recent activities which aim at advancing the field through the overall improvement of the traditional regulatory genotoxicity testing paradigm for better hazard assessment relying on fewer or no animals. It covers the improvement of existing tests, the development of novel assays, as well as approaches to optimise the accuracy of the core testing battery. Tools to support progress in this field, such as the development of a genotoxicity and carcinogenicity database and reference chemical lists are also presented. The approaches described herein will offer solutions in the short- and medium-term while, progress in mechanistic understanding and emerging biomedical technologies will likely provide, in the longer term, opportunities to consider completely new approaches and assessment strategies.

Abbreviations: Carc, carcinogenicity; CAvit, *in vitro* chromosomal aberration test; CAviv, *in vivo* chromosomal aberration test; DB, database; DNAviv, *in vivo* DNA strand breakage (e.g. comet or alkaline elution) assay; EURL ECVAM, EU Reference Laboratory for Alternatives to Animal Testing; GFP, green fluorescence protein; HET-MN, Henn's egg test for micronucleus induction; *Hprt*, hypoxanthine-guanine phosphoribosyl transferase locus; IATA, integrated approach to testing and assessment; MLA/tk, mouse lymphoma *tk* gene mutation assay; MNvit, *in vitro* micronucleus test; MNviv, *in vivo* micronucleus test; NTP, National Toxicology Program; OECD, Organisation for Economic Co-operation and Development; PPP, plant protection products; SCCS, Scientific Committee on Consumer Safety; TG, Test Guideline; TGR, *in vivo* transgenic rodent mutation assay; UDSviv, *in vivo* unscheduled DNA synthesis test.

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2. Regulatory background

Genotoxicity testing includes the measurement of DNA primary damage that can be repaired and is therefore reversible, as well as the detection of stable and irreversible damage (i.e. gene mutations and chromosome aberrations) that is transmissible to the next generation when it occurs in germ cells, and the perturbation in mechanisms involved in the preservation of the integrity of the genome. For an adequate assessment of genotoxicity three major endpoints need to be evaluated (gene mutation, structural chromosome aberrations and numerical chromosome aberrations), as each of these events has been implicated in carcinogenesis and heritable diseases. The standard *in vitro* test battery comprises the bacterial reverse mutation assay (OECD TG 471), the *in vitro* mammalian chromosomal aberration test (OECD TG 473), the *in vitro* mammalian cell gene mutation test (OECD TG 476 [Hprt] and TG 490 [MLA/tk]), and the *in vitro* mammalian cell micronucleus test (OECD TG 487) (Fig. 1).

Any confirmatory *in vivo* follow-up test needs to cover the same endpoint as the one which showed positive results *in vitro*.

Currently, the most commonly used *in vivo* tests comprise the mammalian erythrocyte micronucleus test (OECD TG 474), the mammalian bone marrow chromosomal aberration test (OECD TG 475), the transgenic rodent somatic and germ cell gene mutation assay (OECD TG 488) and the *in vivo* mammalian alkaline comet assay (OECD TG 489). All OECD Test Guidelines (TGs) are available at the OECD website.

The assessment of genotoxic hazard to humans currently follows a stepwise approach, beginning with a basic battery of *in vitro* tests followed in some cases by *in vivo* testing (Fig. 2).

Regulatory requirements, in particular for *in vivo* testing, vary depending on the type of chemical under regulation and the region. For cosmetics, *in vivo* testing is prohibited in the EU (EC, 2009a; SCCS, 2015) while for industrial chemicals and biocidal products a positive outcome in one or more of the *in vitro* genotoxicity tests requires confirmation by appropriate follow-up *in vivo* testing (EC, 2008; EC, 2006; EU, 2012). In these cases, if a substance is clearly negative in the *in vitro* battery it is considered as having no genotoxic hazard, thus no further *in vivo* study is needed. Regulatory requirements for pharmaceuticals, veterinary drugs and plant protection products foresee that the *in vitro* testing battery (irrespective of the outcome) is always followed by *in vivo* testing (ICH S2(R1), 2011; VICH GL23(R), 2014; EC, 2009b and EU, 2013a; b).

Of note, the Committee for Medicinal products for Veterinary Use (CVMP) is looking at opportunities for implementation of the 3Rs, in the case of genotoxicity by considering to remove the default requirement for an *in vivo* test (ei.e. if all *in vitro* results are clearly negative) or to allow this test to be incorporated into another *in vivo* test (such as repeat dose toxicity) (Reflection Paper, 21 April 20161EMA/CHMP/CVMP/JEG-3Rs/164002/2016).

3. Main gaps identified: high rate of misleading/false genotoxic positives

Even though a number of well-established *in vitro* methods are available and officially accepted for genotoxic hazard assessment, at present they cannot be considered to fully replace animal tests (Adler et al., 2011). Therefore, new methods and strategies continue to be developed. This is because the existing *in vitro* methods, whilst having a high sensitivity (and thus low false negative rate), have a relatively low specificity and thus high rate of false ("misleading") positive results, which typically leads to unnecessary follow-up testing *in vivo* for the confirmation of these results (Kirkland et al., 2005). During a workshop organised by EURL ECVAM (2006, Ispra, Italy) the high rate of unexpected positive results in *in vitro* mammalian cell genotoxicity tests was addressed (Kirkland et al., 2007). It was recommended that better guidance on the likely mechanisms resulting in positive results not relevant for humans, and how to obtain evidence for these mechanisms was needed. The workshop recommendations have contributed to several international collaborative initiatives aiming to improve the existing genotoxicity *in vitro* tests and to identify and evaluate new cell systems with appropriate sensitivity but improved specificity. This resulted in the identification of several reasons for misleading positives. In fact, it is now known that it is possible to limit these misleading positives by: 1) using p53-competent human cells; 2) choosing measures of cytotoxicity based on cell proliferation; 3) carefully checking the source and the characterisation of the cells; 4) testing at reduced maximum concentration (Parry et al., 2010; Kirkland and Fowler, 2010; Fowler et al., 2012a, 2012b).

The knowledge acquired during the last decade of testing has already been considered in the recent revision of OECD Test Guidelines (TGs) for genotoxicity and in the Guidance Document on OECD Genetic Toxicity TGs (OECD website). The revised TGs are expected to enhance the quality of the data produced and consequently avoid in some cases the need for *in vivo* confirmation of the results. In an attempt to enhance testing harmonisation, some of the revisions are cross-cutting through the different *in vitro* mammalian cell genotoxicity TGs. These include a highest tested concentration of 10 mM, 2 mg/mL or 2 μL/ml, whichever is the

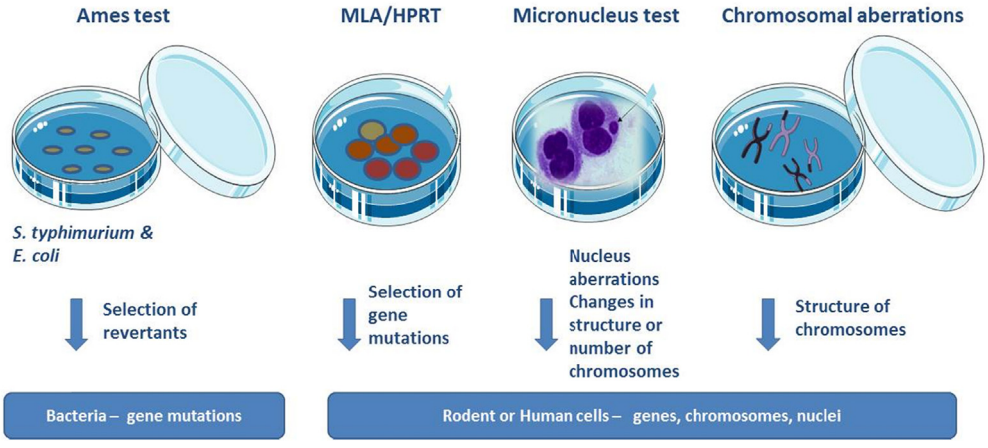


Fig. 1. *In vitro* test battery.

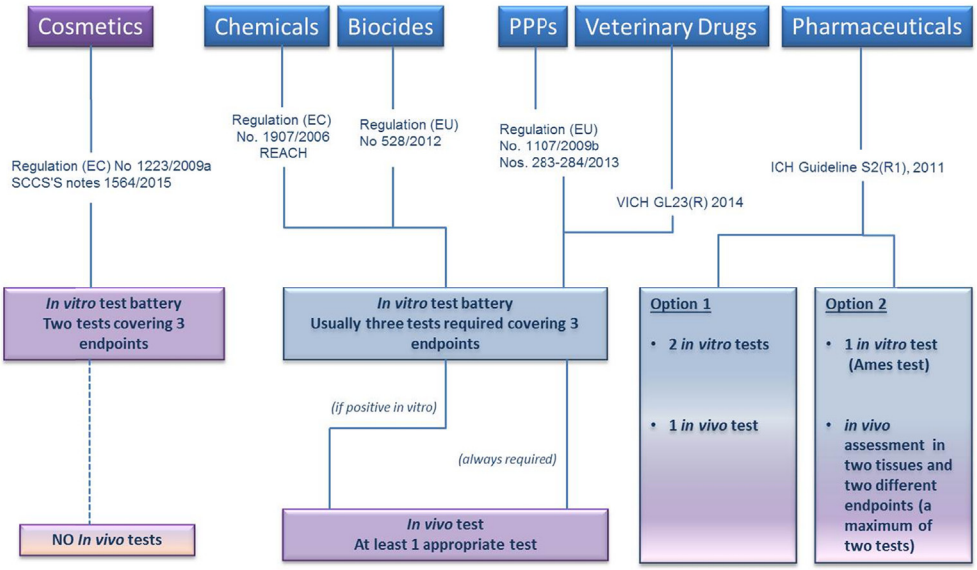


Fig. 2. Summary of testing requirements and guidance documents for genotoxicity assessment in the EU.

lowest (instead of 5 mg/mL) if toxicity and solubility are not limiting factors; more guidance on appropriate cytotoxicity measures to use (e.g. measurements of cell proliferation for the micronucleus test and the chromosome aberration assay); the demonstration of proficiency of the laboratory conducting the test (comprising a list of chemicals included in the TGs); recommendations for the establishment and use of historical controls; and harmonisation of data interpretation for OECD TGs 487, 473 and 476 (OECD website).

4. Can the performance of genotoxicity testing be enhanced?

Several options are currently being explored to improve the overall assessment of genotoxicity. A strategic plan to avoid and reduce animal use in genotoxicity testing had previously been described by EURL ECVAM, based on the regulatory needs across different EU legislations, state of the science, and latest and ongoing efforts undertaken by various organisations, including EURL ECVAM (ECVAM, 2013) (Fig. 3).

It was proposed that efforts should be directed towards the overall improvement of the current genotoxicity testing strategy for better hazard assessment with the use of fewer or no animals to satisfy the information requirements of various EU regulations.

This involved the pursuit of two key aims. The first strategic aim focused at enhancing the performance of the *in vitro* testing battery to reduce the need for *in vivo* follow-up tests.

It applies to the base set of the *in vitro* testing battery (e.g. best tests combination, improvement of single tests), as well as to supplementary tests or non-testing methods used to confirm relevance of positive results (e.g. identify mechanisms of action).

The analysis of the most suitable combinations of *in vitro* genotoxicity tests is a key consideration for possible improvements of genotoxicity testing strategies. Some studies addressing this issue are described in the following sections.

Since *in vivo* genotoxicity testing is still a requirement to confirm *in vitro* results in most regulatory contexts it was considered important in the second strategic aim to focus on the reduction and optimisation of the use of animals during *in vivo* testing. Several opportunities for reduction exist both at single test level (e.g. 1 sex versus 2 sexes, smaller animal groups) and at integrated strategy level (Pfuhler et al., 2009; EFSA, 2011). The integration of different endpoints into a single study (Pfuhler et al., 2009; Bowen et al., 2011) or the incorporation of *in vivo* genotoxicity endpoints into a short-term repeated dose toxicity test (28 days) (Rothfuss et al., 2010, 2011; EFSA, 2011), if such a test is going to be performed anyhow, should always be considered. Most of the currently accepted *in vivo* tests are amenable to such integration. An integration of genotoxicity endpoints offers the possibility for an improved interpretation of genotoxicity findings since these data will be evaluated in conjunction with routine toxicological information obtained in the repeated dose toxicity study, such as: haematology, clinical chemistry, histopathology and exposure data. Moreover, the selection of the appropriate follow-up *in vivo* test is important, especially now that additional *in vivo* genotoxicity OECD TGs have been adopted (OECD TG 488, on transgenic rodent somatic and germ cell gene mutation assays and OECD TG 489, on *in vivo* mammalian alkaline comet assay). The ILSI HESI Genetic Toxicology Technical Committee is currently collecting data in order to assess which *in vivo* test is the most appropriate test to follow-up *in vitro* genotoxicity tests.

5. Optimal number of tests in the core *in vitro* battery

Testing requirements across different regulatory sectors have recommended a three *in vitro* test battery comprising a test for induction of gene mutations in bacteria, a test for induction of gene mutations in mammalian cells, and an *in vitro* chromosomal aberration or micronucleus test. The principles behind the use of such battery are to detect gene mutation, structural and numerical chromosomal damage. A question that was posed in the past years was whether it is necessary to include two mammalian cell tests in order to achieve the coverage of the three endpoints. If both bacterial and mammalian cell tests for gene mutation are conducted, and the results generated are discordant (i.e. one is positive and the other negative), it is questionable which test should take precedence.

With the aim of improving the assessment of genotoxicity, especially in relation to the reduction of false positives and consequently of unnecessary follow-up animal tests, analyses have been conducted on the possibility of modifying the core *in vitro*

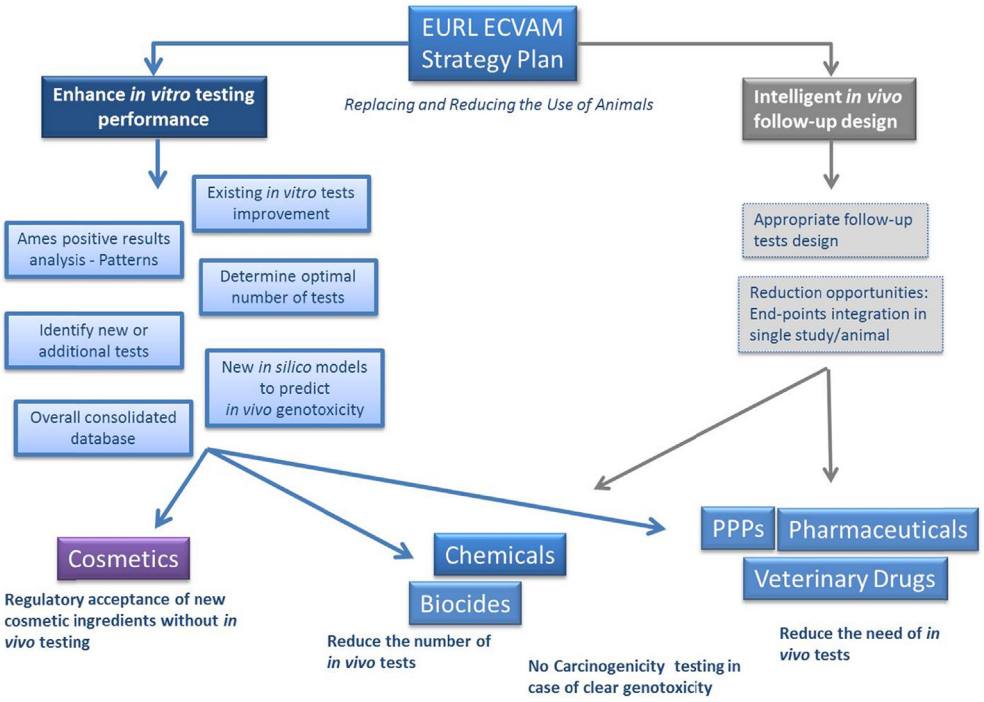


Fig. 3. Efforts to replace and reduce the use of animals in genotoxicity testing.

testing battery. A previous evaluation of the results from combinations of two or three assays had shown that the sensitivity increases whereas the specificity decreases when more tests are combined (Kirkland et al., 2005). For example, the combination of three tests, including the mouse lymphoma assay, which measures gene mutations in mammalian cells (and can in some cases be indicative of structural chromosome aberrations), had a slightly higher sensitivity but the specificity further decreased compared with a combination of two tests. It would appear that a strategy of three tests is not more accurate to identify chemicals of genotoxic concern than one based on two tests, although it is generally felt to be "safer" because of the additional study performed.

In a subsequent analysis of an existing database of rodent carcinogens and an additional database of *in vivo* genotoxins, together covering over 950 substances, Kirkland et al. (2011) confirmed that data from the gene mutation test in bacteria and the *in vitro* micronucleus test allow the detection of all the relevant *in vivo* carcinogens and *in vivo* genotoxins for which data exist in these databases considered. Consequently, it would appear that the core battery could consist of a combination of two *in vitro* tests.

For an adequate evaluation of the genotoxic potential of a chemical substance, there is general agreement that the basic requirements should cover the three endpoints of genotoxicity. Therefore, assuming the choice of the Ames test to identify gene mutations, as one of the tests, the only option for two tests which cover the three endpoints is a combination of the Ames test with the *in vitro* micronucleus test. The bacterial reverse mutation assay detects gene mutations and the *in vitro* micronucleus test detects both structural and numerical chromosome aberrations. Ultimately, the two test combination versus the three test battery may lead to a reduction in the number of unnecessary follow-up animal tests, as well as a reduction of costs, retaining nevertheless an adequate sensitivity.

In regard of a newer approach for genotoxicity testing, in relation to the core *in vitro* battery, several authoritative documents have been published in recent years, which give guidance on how different types of substances can be assessed for genotoxicity. Specifically, the use of a core battery composed of two *in vitro* tests, the *in vitro* bacteria test and the *in vitro* micronucleus test, has been advocated by the following Committees: 1) UK Committee On Mutagenicity (COM) in a guidance for genotoxicity testing of chemical substances (COM, 2011); 2) EFSA Scientific Committee in their scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment (EFSA, 2011); 3) Scientific Committee on Consumer Safety (SSCS) notes of guidance for the testing of cosmetic ingredients and their safety evaluation, 9th revision (SCCS/1564/15). These documents currently guide the hazard and risk assessment of substances in food and feed and cosmetics ingredients in the EU, which is carried out by the EFSA Scientific Panels and the SCCS Scientific Committee, respectively.

6. Are there categories of positive results from the Ames test that are irrelevant or signify low risk?

Differently from *in vitro* mammalian cell tests, no systematic analysis on Ames test accuracy was conducted until recently, despite the Ames test being the primary test for genotoxicity screening and the most used test within the *in vitro* battery. It has been shown that positive results in the Ames test correlate well with carcinogenic potential in rodents but, not perfectly, being mutations only one of many events in tumor development. Since most chemicals are also tested for genotoxicity in mammalian cells, the pattern of mammalian cell results may help identify whether Ames test positive chemicals predict carcinogenic or *in vivo* mutagenic activity. Therefore, a workshop was organized by EURL ECVAM to investigate whether the *in vitro* mammalian cell genotoxicity test results could complement and mitigate the implications of a positive Ames test response for the prediction of *in vivo* genotoxicity and carcinogenicity, and if patterns of results could be identified (Kirkland et al., 2014a). Participants from regulatory agencies, academia and industry presented results from *in vitro* mammalian cell genotoxicity tests associated with Ames-positive

compounds. The question was posed whether negative results in mammalian cell tests were associated with absence of carcinogenic or *in vivo* genotoxic activity despite a positive Ames test. Possible reasons why a positive Ames test may not be associated with *in vivo* activity and what additional investigations/tests might contribute to a more robust evaluation were also discussed. For instance, situations can be envisaged where the mutagenic response may be specific to the bacteria or the test protocol, such as, bacterial-specific metabolism, exceeding a detoxification threshold, or the induction of oxidative damage to which bacteria may be more sensitive than mammalian cells *in vitro* or tissues *in vivo*. A considerable overlap was identified among the different databases presented. It was therefore recommended that a consolidated database be built, to avoid data duplication, so that a more robust analysis of the predictive capacity for potential carcinogenic and *in vivo* genotoxic activity could be derived from the patterns of mammalian cell test results obtained for Ames-positive compounds (see Section 7 for the detailed description of the consolidated database). The data collected have been analyzed by using different approaches (Kirkland et al., 2014b). The first approach measured positive and negative predictive values. A positive predictive value determines the probability that positive results in the Ames and mammalian cell tests are indicative of *in vivo* genotoxic or carcinogenic activity, and the negative predictive value, the opposite. The second approach measured sensitivity and specificity. Sensitivity determines the frequency with which *in vivo* genotoxins and carcinogens give positive results in both Ames and mammalian cell test; while specificity determines the frequency with which chemicals that are positive in the Ames test but, not genotoxic *in vivo* or carcinogenic, give negative results in mammalian cell tests. Finally, the concordance between *in vitro* and *in vivo* data was also evaluated, where, the level of agreement of results between *in vitro* and *in vivo* tests from the same endpoints was analysed. All the above approaches provided similar results. It is worth noting that, because the database was limited to Ames-positive chemicals, the majority (>85%) of carcinogens and *in vivo* genotoxins were positive when tested in both *in vitro* gene mutation and aneugenicity/clastogenicity tests. However, about half (>45%) of chemicals that were not carcinogenic or genotoxic *in vivo* also gave the same patterns of positive mammalian cell results. Although the different frequencies were statistically significant, positive results in two *in vitro* mammalian cell tests did not, *per se*, add to the predictivity of the positive Ames test. By contrast, negative results for both *in vitro* mammalian cell endpoints were rare for Ames-positive carcinogens and *in vivo* genotoxins but, were significantly more frequent for Ames-positive chemicals that are not carcinogenic or genotoxic *in vivo*. Thus, in the case of an Ames-positive chemical, negative results in two *in vitro* mammalian cell tests covering both mutation and clastogenicity/aneugenicity endpoints should be considered as indicative of absence of *in vivo* genotoxic or carcinogenic potential (Kirkland et al., 2014b). Surely, it would be inappropriate to rely exclusively on negative results from *in vitro* mammalian cell tests or *in vivo* bone marrow chromosomal damage tests to conclude that an Ames test positive chemical would not possess carcinogenic or *in vivo* genotoxic activity. Investigations as to why an Ames test might be a false positive would be useful. Furthermore, follow-up *in vitro* tests (e.g. additional *in vitro* assays, gene expression profiles, cell transformation assays, etc.) might aid significantly the interpretation of the relevance for humans of the *in vitro* genotoxicity results vis-à-vis *in vivo* genotoxicity or carcinogenicity. Such an approach is described in the recent revision of the SCCS's Notes of Guidance for testing cosmetic ingredients and their safety evaluation in the area of mutagenicity and genotoxicity where the outcome of this analysis has been considered (SCCS, 2015) and by Luijten and colleagues in a proposed integrative test strategy for cancer hazard identification (Luijten et al., 2016).

7. EURL ECVAM Genotoxicity & Carcinogenicity Consolidated database

The Genotoxicity & Carcinogenicity Consolidated database (DB) constructed following the recommendation of the EURL ECVAM workshop (see Section 6) was launched at the end of 2014 (https://eurl-ecvam.jrc.ec.europa.eu/databases/genotoxicity-carcinogenicity-db).

It represents a structured master database that compiles available genotoxicity and carcinogenicity data for Ames positive chemicals originating from different sources and complemented by literature search. The main sources considered were: EU DBs (EFSA, ECHA, SCCS); international DBs (Jap ISHL, Jap CSCL, US NTP); literature DBs (Kirkland et al., 2005, 2011; IssTox) and industry ones (Cosmetic ingredients, Chemicals, Pharmaceuticals) (Fig. 4).

A total of 937 compounds were firstly selected (Fig. 5). By using a harmonized format to capture the information, (comparison among different databases, elimination of replicates and review of each single test) the resulting collection of 726 unique chemicals, with a total of >5500 entries and >250 references, included results from each single database consulted and overall calls for each endpoint for each chemical.

In addition, a rigorous methodology and defined criteria were applied for the selection and analysis of the data:

a. Selection of compounds with a known chemical identity (structure, purity, molecular weight, CAS number).
b. Selection of compounds with valid *in vitro* and *in vivo* results for the genotoxicity endpoints or for carcinogenicity. Data were collected for the following tests: *in vitro* tests (Ames, mouse lymphoma Tk+/- [MLA] or Hprt, micronucleus [MN], chromosome aberration [CA]); *in vivo* tests (MN, CA, UDS, transgenic models [TGR], DNA breakage [Comet and alkaline elution assay]); rodent carcinogenicity.
c. Combination into single entries of free bases and simple acid salts or *R*- and *S*-isomers for those chemical substances where a similar behavior was expected and/or proven.
d. "Overall Call" definition for each genotoxicity endpoint *in vitro* and *in vivo* and carcinogenicity by following defined criteria for the reliability of each study and quality of data for those chemicals appearing in more than one source with different calls. Four categories were considered positive [+], negative [−], equivocal [E] and inconclusive [I]. Where information was missing, even for those chemicals with one single data entry, scientific literature was consulted (Kirkland et al., 2014a).

It is worth noting that the database not only serves as a resource for evaluating the predictivity of the Ames test for *in vivo* genotoxicity and carcinogenicity when considered alone or in association with *in vitro* mammalian cell assays and for a better characterisation of those cases where the Ames test leads to irrelevant results. Rather, it has become an important source of consultation for the genotoxicity regulatory and scientific community. For instance, it has been used by: ECHA in view of supporting the evaluation of genotoxicity data for the 2018 REACH deadline; 2) by the CEFIC Long-Range Research Initiative (LRI-B18) as the starting point for a broader project aimed at constructing a database on carcinogen dose-response for threshold of toxicological concern (TTC) analysis to be used as part of the safety regulatory assessment. It also recently has been used to investigate the performance of an integrated approach to testing and assessment (IATA) designed to cover different genotoxic mechanisms causing cancer (Petkov et al., 2016). Furthermore, the database may be

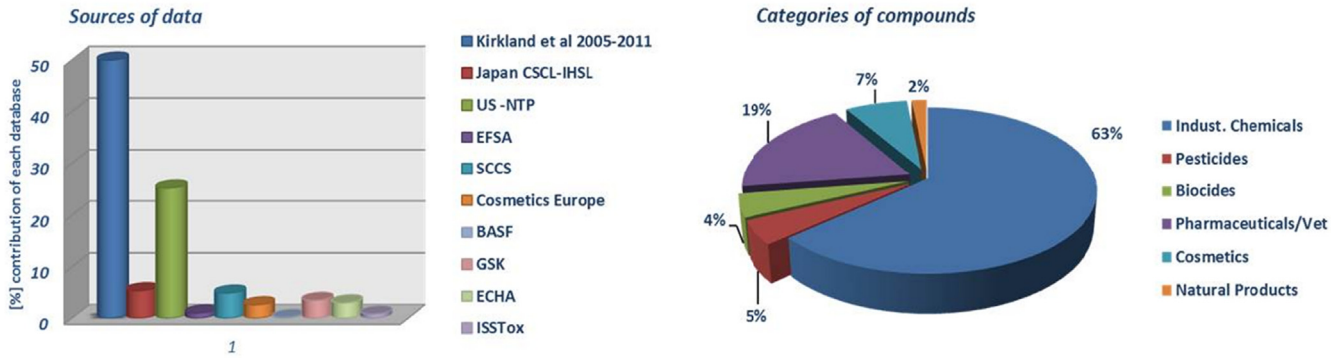
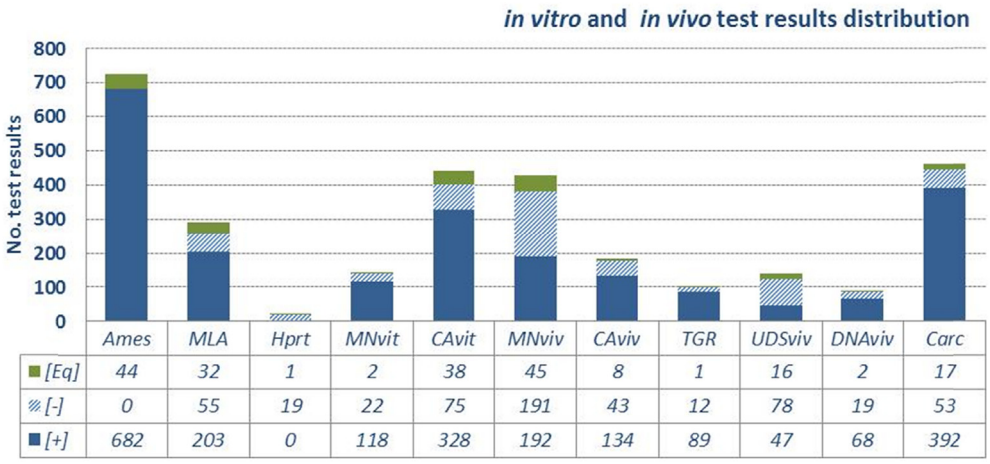


Fig. 4. Source of data within the EURL ECVAM consolidated database.



	Ames	MLA	Hprt	MNvit	CAvit	MNviv	CAviv	TGR	UDSviv	DNAviv	Carc
[Eq]	44	32	1	2	38	45	8	1	16	2	17
[-]	0	55	19	22	75	191	43	12	78	19	53
[+]	682	203	0	118	328	192	134	89	47	68	392

Fig. 5. Distribution of results from the EURL ECVAM consolidated database. The table reports data for [+] positive, [−] negative and [Eq] equivocal Ames results.

utilised as a platform for detailed structural characterization of specific groups of compounds with or without carcinogenic or genotoxic activity (e.g. analysis of organic functional groups using the OECD Tool Box). For this purpose, the database has been linked to two other platforms, developed by the EC Joint Research Centre, the CHEList and CHemAgora, which provide a means of identifying whether a chemical has been used in previous research or validation projects (including EU-funded, international and JRC projects) and whether the chemical of interest is regulated and listed under a specific regulatory inventory. These platforms also provide direct links to information included in third party databases. The database is currently hosted by EURL ECVAM, at: https://eurl-ecvam.jrc.ec.europa.eu/databases/genotoxicity-carcinogenicity-db though, a website migration to the JRC Science Hub https://ec.europa.eu/jrc/ is foreseen by the second half of 2016. The database is to be considered a living project with possibilities of update and expansion. In fact, a project aimed at extending the database to include Ames negative chemicals is currently ongoing.

8. Efforts to develop new *in vitro* methods

Alongside the development of curated databases and the improvement of the accuracy of existing *in vitro* methods several activities are currently in place with the aim of exploring new areas of development. For instance, the micronucleus test and the comet assay in 3D human reconstructed skin models offer the potential for a more physiologically relevant approach especially regarding metabolic aspects to test dermally applied chemicals (Hu et al., 2010; Pfuhler et al., 2011). Validation studies of the micronucleus test using the human reconstructed skin models are under finalisation by Cosmetics Europe, and in the case of the 3D skin comet assay, by a joint effort between Cosmetics Europe and a German Consortium funded by BMBF (Aardema et al., 2010; Reus et al., 2013). The features of the reconstructed skin models have been suggested to improve the predictive value of a genotoxicity assessment compared with that of existing *in vitro* tests.

Another promising system, proposed as a follow-up for *in vitro* positives, is the hen's egg test for micronucleus induction (Wolf et al., 2008). Although it is not a human-based system, the HET-MN combines the use of the commonly accepted genetic endpoint “formation of micronuclei” with the well-characterised and complex model of the incubated hen's egg, which enables metabolic activation, elimination and excretion of xenobiotics, including those that are mutagens or pro-mutagens. The transferability and intra-/inter-laboratory reproducibility are currently being evaluated (Greywe et al., 2012).

In vitro toxicogenomics-based tests can inform on the specific mode of action of a potential genotoxicant early on in development. Toxicogenomics identifies global gene expression changes associated with a toxicological outcome. In the context of genotoxicity testing, its primary use is envisaged to be in providing information to be used as a potential mechanistically based follow-up test for positive *in vitro* genotoxicity results (Doktorova et al., 2014; Luijten et al., 2016). In fact, common features emerge with respect to those molecular pathways underlying *in vitro* and *in vivo* results. At the moment, these tests are in use to generate supportive mechanistic

information rather than for routine testing. Interestingly, an *in vitro* toxicogenomic biomarker assay is being validated by the HESI Genomics Committee and is foreseen to undergo the FDA biomarker qualification process (Li et al., 2015; Buick et al., 2015). Recently, gene expression profiles have also been proposed to be useful tools in human health risk assessment providing not only qualitative, but also quantitative information in relation to the relevant mode of action induced by the compound (Moffat et al., 2015; Bourdon-Lacombe et al., 2015).

In the last few years several attempts have been made to develop high throughput genotoxicity screening assays by using the induction of stress pathways/proteins as endpoints. The choice of the pathways was mostly based on microarray experiments with genotoxic substances. The GreenScreen HC assay, which uses a p53-competent TK6 lymphoblastoid cell line genetically modified to incorporate a fusion cassette containing the GADD45alfa promoter and the GFP gene as reporter, has been widely characterised (Hastwell et al., 2006, 2009; Jagger et al., 2009; Birrell et al., 2010). Other high throughput assays based on DNA damage response pathways established in various cell lines have shown to be useful for screening in early phases of drug development with the potential to reduce the attrition rate due to genotoxicity (e.g. Westerink et al., 2010; Khoury et al., 2013; Garcia-Canton et al., 2013; van der Linden et al., 2014). More recently, assays that simultaneously analyse different biomarkers (e.g. p53, gH2AX, p-H3 or polyploidy), including cellular responses to DNA damage as well as overt cytotoxicity, have been developed to provide a more mechanistical information on the types of biological damage induced by different classes of substances (Hendriks et al., 2016; Bryce et al., 2016). Also, assays based on cell lines and primary cells derived from transgenic rodents are in use, which can originate from different tissues and may reduce assumptions related to extrapolation from *in vitro* to *in vivo* tests as they assess exactly the same endpoint and marker genes as the respective *in vivo* transgenic models (Berndt-Weis et al., 2009; Zwart et al., 2012).

9. Recommended list of chemicals to assess the performance of new or improved genotoxicity tests

Reference chemical selection is a key step in the development, optimisation and validation of alternative test methods. A first reference list, of genotoxic and non-genotoxic chemicals, published in 2008 (Kirkland et al., 2008), has become an internationally recognized resource for scientists and has been used for a variety of purposes, including the development of new assays, the optimisation of existing test protocols, the implementation of automated high throughput assays and the design of validation studies. In addition, the reference list has proven invaluable in the attempt to reduce misleading positive results obtained from some *in vitro* methods.

In light of newly available data, it was considered appropriate to update this list of genotoxic and non-genotoxic chemicals recommended for assessing the performance of new or improved *in vitro* genotoxicity test methods to fit the following different sets of characteristics (Kirkland et al., 2016):

Group 1: Chemicals that should be detected as positive in *in vitro* mammalian cell genotoxicity tests. Chemicals in this group are all *in vivo* genotoxins at one or more endpoints, either due to DNA-reactive or non DNA-reactive mechanisms. Many are known carcinogens with a mutagenic mode of action, but a sub-class of probable aneugens has been introduced.

Group 2: Chemicals that should give negative results in *in vitro* mammalian cell genotoxicity tests. Chemicals in this group are usually negative *in vivo* and non-DNA-reactive. They are either non-carcinogenic or rodent carcinogens with a non-mutagenic mode of action.

Group 3: Chemicals that should give negative results in *in vitro* mammalian cell genotoxicity tests, but have been reported to induce gene mutations in mouse lymphoma cells, chromosomal aberrations or micronuclei, often at high concentrations or at high levels of cytotoxicity. Chemicals in this group are generally negative *in vivo* and negative in the Ames test. They are either non-carcinogenic or rodent carcinogens with an accepted non-mutagenic mode of action. This group contains comments as to any conditions that can be identified under which misleading positive results are likely to occur.

The revised list contains a total of 69 chemicals of different structural classes and modes of action, grouped according to whether positive or negative results are expected when tested *in vitro*. The list also includes chemicals that are currently suspected of generating “misleading” or “irrelevant” positive results in some existing assays. The recommended list and the supporting data are expected to make an important contribution to the development and acceptance of new and refined *in vitro* genotoxicity test methods with improved predictivity and technical performance.

10. Future applications

Conventional test methods have evolved and have been modified for specificity, sensitivity and high throughput capacity. Moreover, novel *in vitro* methods are being developed which are indeed promising. Although they can already be used to better understand modes of action, judge the relevance of the data obtained with the standard assays (e.g. differentiating DNA-reactive from DNA-non-reactive compounds), and to help predict *in vivo* effects when animals cannot be used, they yet do not allow for a complete replacement of current regulatory tests across all sectors.

Other emerging technologies and non-classical methodologies, as high throughput assays, computational approaches, coupled with novel *in vitro* model systems and sequencing (Zhang et al., 2014), may ultimately drive the development of completely new integrated approaches to testing and assessment of genotoxicity, which break away from the paradigm established over the past 40 years of regulatory testing. However more research, development and data integration efforts are still required before more 'radical' solutions emerge which can stand up to the rigorous demands of regulatory testing. In the short to medium term therefore, reduction and possibly replacement of animal testing for genotoxicity assessment is most likely achievable through a pragmatic approach of using sound scientific rationale to improve the current testing paradigm, in a manner acceptable to both the regulatory and regulated communities.

Conflict of interest

The authors declare that there are no conflicts of interest.

Notes

The authors declare no competing financial interest.

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