the work described. The most current version of the software at publication is DX version 4.1.0.

Curation and combination of data sets

The three data sets of *in vivo* chromosome damage data [comprised of both micronucleus (MN) test and chromosome aberration (CA) test data] outlined below were used in this work:

MMS data set—compiled and provided by the MMS group of Japan. Data set contained a total of 255 compounds with associated *in vivo* MN test data. An overall call for each compound had been made based on expert interpretation of the primary data. One hundred twelve compounds had been assigned a call of positive, 129 negative and 14 inconclusive.

FDA data set—*in vivo* chromosome damage data originating from the FDA of the USA. Data set contained a total of 939 compounds with associated *in vivo* MN and/or CA test data. The overall calls for MN and CA for each compound were generated by Leadscope Enterprises (Leadscope Incorporated, http://www.leadscope.com/) on behalf of the FDA and were used. An overall call for *in vivo* chromosome damage was generated by combining these results where necessary. A positive result in either test meant the assignment of an overall call of positive. If the compound did not give a positive result but produced an equivocal result in either test it was assigned as equivocal. Otherwise the result was given as negative. For compounds which had only been tested in a single assay the result in a single test was used as the basis of the overall call. Two hundred eighty-eight compounds were assigned a call of positive, 625 negative, 13 equivocal and 13 inconclusive.

Vitic Nexus data set—extracted from the online database Vitic Nexus (database version 1.75). Data set contained a total of 804 compounds with associated *in vivo* MN test data. An overall call for each compound was generated based on the individual study data. A positive call in any study was used to assign an overall call of positive. Compounds which did not give a positive result but with a 'weak positive' call in any study were assigned as weakly positive. Compounds without any positive calls but with an equivocal call in any study were assigned as equivocal and those which only had negative results in all the studies carried out were assigned an overall call of negative. Two hundred forty-one compounds were assigned a call of positive, 503 negative, 3 weakly positive, 25 equivocal and 32 inconclusive.

A combined data set generated from these three smaller data sets with an overall activity for each compound was then produced. Structures present in more than one data set were identified based on matching chemical structures or chemical abstracts service (CAS) numbers. An analysis of the overlap between the different data sets and the concordance in results between these is shown in Figure 2. An overall call for the duplicate structures was assigned based on the following criteria. If the compound gave a positive result in any one of the data sets it was assigned a positive overall. Failing this if the compound was assigned as weakly positive in any data set it was assigned weakly positive. If a compound had not been assigned either of these results in any data set but had been assigned as equivocal in a data set it was assigned equivocal. Otherwise the compound was assigned as negative unless it had only been tested in one data set and in this case the single alternative assignment was used (e.g. inconclusive). This conservative approach was felt to be sufficient in activity assignment for lead generation since any discordant data would be further resolved at a later point by expert consideration of the primary data. Following this combination, compounds with a result other than positive or negative were removed from the data set. The combined data set produced consists of 1461 compounds with 484 positive and 977 negative compounds. This combined data set was then used to facilitate subsequent analysis of the *in vitro* alerts.

Identification of candidate alerts

The combined data set was processed against the existing *in vitro* chromosome damage alerts in Derek Nexus (Derek Nexus version 2.0) and positive predictivity (PP) values calculated for each alert.

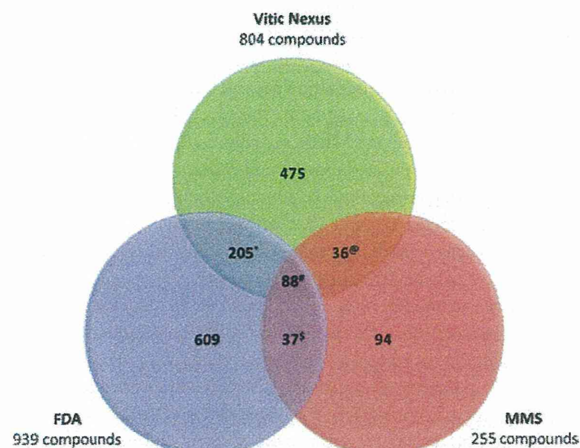

Figure 2. Venn diagram showing data set overlap. Overlap between the Vitic Nexus and MMS data sets was assessed by matching CAS numbers. Overlap with the FDA data set was assessed by duplicate structure matching in Instant JChem. Call agreement between each data set is: #53% have three calls the same, 43% two calls the same, 3% all disagree; @69% calls agree, 31% disagree; *82% agree, 18% disagree; $68% agree, 32% disagree.

Alerts were then prioritised based on this list. The alerts selected were those which were thought to be good candidates for exploration regarding their applicability to *in vivo* test systems. One objective of this work was to increase the predictivity of the endpoint of *in vivo* chromosome damage in Derek Nexus and create a more comprehensive knowledge-based expert system for the endpoint. With this in mind alerts with a PP > 50% and with >2 total activating compounds were prioritised (good overlap between the *in vivo* and *in vitro* endpoints) for further investigation. Cases where more than 20 compounds activated the alert were also prioritised (a significant amount of data with which to make assessment of the *in vivo* activity of this class of compound) for further analysis.

All of the 24 alerts identified which met these criteria were then investigated by a human expert in the endpoint of *in vivo* chromosome damage. In addition, eight alerts which did not meet the criteria outlined above but were identified as good candidates were also investigated further as part of this work.

Development of in vivo chromosome damage predictions

For each of the 32 alerts investigated, all the *in vivo* data available in the data set were examined in detail referring back to the original citations where possible. In addition to data from the combined data set used in the prioritisation, searches for extra data from the publically available scientific literature were also performed. These searches were initially made of large toxicologically relevant databases such as PubMed (http://www.ncbi.nlm.nih.gov/pubmed) and CCRIS using ChemID*plus* (http://chem.sis.nlm.nih.gov/chemidplus/). Wider, more detailed searches were then made based on the findings from these sources. Both data from *in vivo* chromosome damage assays as well as information relating to potential reasons for activity and mechanism by which a compound class may cause activity were gathered. Once all of the available relevant information on the compound class had been collected it was collated and an overall conclusion on the activity of each compound was drawn by the expert. When drawing these conclusions, consideration was made on the quality of the test carried out (compliance with good laboratory practice) as well as the relevance of the protocol employed based on the likely absorption, distribution,

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metabolism and excretion (ADME) properties of the compound and the mechanism which may cause chromosome damage. Data were taken from the *in vivo* micronucleus and chromosome aberration tests. Data from the Comet assay were not used since this test measures mutational events and general DNA damage rather than the ability of a chemical to induce chromosome damage. Where possible, conflicting results for the same compound were justified based on differences in test protocol (e.g. dose, sampling time, tissue, route of administration, etc.).

With all available data in hand, a decision was made regarding the relevance of each *in vitro* alert to *in vivo* activity. In cases where the *in vitro* alert was believed to be predictive of *in vivo* activity, the alert was extended to predict both endpoints. A new reasoning rule was added to the alert, set at the likelihood of either plausible or equivocal depending on the strength of evidence in support. The alert comments were updated and supporting references and examples added where appropriate. For those alerts where there was not a good overlap between the activity seen in *in vitro* systems and *in vivo* systems an assessment was made to determine whether there were particular conditions under which activity could be observed for this compound class or if a particular subclass could be identified. If either of these conditions were met the knowledge base was updated appropriately, in the case of the former the alert comments described the requirements appropriately and in the latter a new alert was implemented for the subclass. This analysis in particular highlights a case where human expert interpretation can be used to find patterns in the data that would be hard to identify using other modelling techniques. Where appropriate these observations can then be encoded in the knowledge base of the expert system, helping the user to make their decision regarding the activity of their query compound. In cases where an *in vitro* alert predicted *in vivo* results poorly and where the above criteria were not met no extrapolation to an *in vivo* prediction was made.

In all the cases outlined above, information on the key data used to make the decision was included in the comments associated with the alert. Any protocol specificity observed or which may be expected based on mechanism leading to toxicity following an expert interpretation, was also included in order to help direct testing. A simplified version of the decision making process used to reach these conclusions is outlined in the decision tree illustrated in Figure 3. This decision tree was used in combination with an expert assessment of the current knowledge relating to the genotoxic activity of each compound class (including information relating to mechanistic rationale where available) in order to reach a conclusion on the best approach to predicting the activity of each class.

Results

Prior to this study, there was limited coverage of *in vivo* chromosome damage in Derek, with only 10 alerts for this endpoint in the knowledge base. The chemical space covered by these alerts was limited and this was reflected by the low sensitivity of Derek against a data set provided by the MMS of Japan, as shown in Table 1.

During this investigation, 32 *in vitro* chromosome damage alerts were investigated. Of the investigated alerts, 19 fulfilled the criteria for extension to cover *in vivo* activity: these alerts were sufficiently predictive of *in vivo* data and it was concluded that *in vitro* and *in vivo* endpoints shared a common mechanism. New reasoning rules were added to these alerts, set at a likelihood of either plausible (nine alerts) or equivocal (10 alerts) depending on the weight of evidence supporting *in vivo* activity (Figure 3, outcomes 1 and 2) (9,10). In some of the cases investigated, *in vivo* activity did not initially appear to reflect *in vitro* findings. However, an expert analysis of the data and mechanism revealed that activity may be dependent on the test protocol used and if certain conditions were employed a positive result *in vivo* would be likely to be obtained for a compound from the class. In these cases a prediction for *in vivo* chromosome damage could still be made which included an explanation of the rationale behind the alert and any specific information pertaining to the most sensitive method of testing the compound and obtaining a positive result.

Additionally, there were instances where a certain subclass of a particular set of chemicals would be likely to show activity *in vivo*

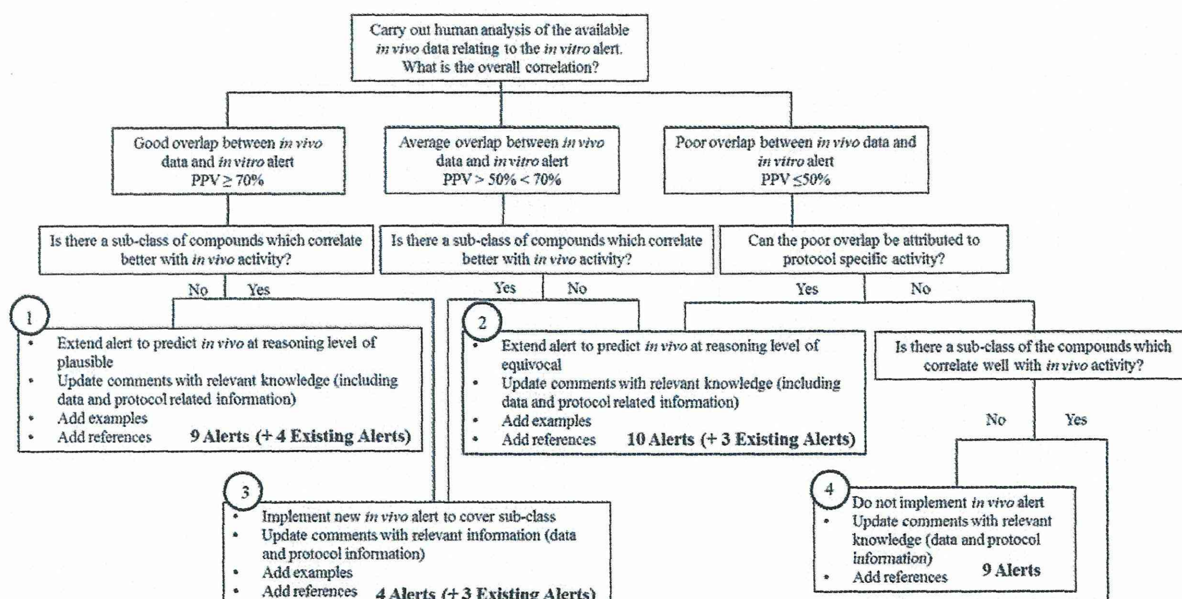

Figure 3. Decision tree representing a simplified version of the decision-making process used determine the updates made to the knowledge base in response to *in vivo* findings.

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while others would not and the differences in activity could be rationalised. In the four cases where this happened the alert comments of the *in vitro* alert were updated and a new alert covering the *in vivo* positive subset was implemented (Figure 3, outcome 3).

Finally, there were nine alerts where *in vitro* activity did not correlate well with *in vivo* activity and no assay specificity could be found or there were compelling reasons why activity may not be seen *in vivo* for the compound class based on mechanism or ADME. In these cases a prediction for *in vivo* chromosome damage could not be made. However, information on the data available or reasons for inactivity were still included in the information of the associated *in vitro* alert (Figure 3, outcome 4).

The results of this work mean that there are now 33 alerts relating to the endpoint of *in vivo* chromosome damage in the Derek Nexus knowledge base (10 existing alerts plus the 23 new alerts developed in this work). Additionally, nine alerts for *in vitro* chromosome damage have now been updated to include information on their relevance to *in vivo* activity.

Discussion

By using a knowledge-based expert approach for the prediction of *in vivo* chromosome damage, human analysis could be used to interrogate the available data and derive an expert conclusion. The value in this analysis is highlighted by the fact that in the three data sets used where a compound was present in more than one data set there was a disagreement in results obtained 18–46% of the time (Figure 2). Expert analysis of these results allowed for a satisfactory resolution of these conflicts. It was also possible to capture all of this information in the knowledge base comprising the predictive system. Information relating to the data available to make a prediction for a given chemical class along with potential mechanism leading to activity and any protocol specificity observed or expected or reasons for likely inactivity was provided (Figure 4). In addition, the activity of similar example compounds were provided where appropriate. Some examples of alerts where the information captured may prove particularly useful are discussed below.

Examples of information provided with structural alerts

Aromatic nitro compounds may display tissue-specific activity

The *in vitro* alert for aromatic nitro compounds demonstrated a low PP (23%) against the *in vivo* data. However, a closer inspection of the *in vivo* data revealed that while aromatic nitro compounds are generally inactive in the rodent bone marrow (BM) micronucleus

Table 1. Predictive performance of Derek for windows version 13 against the MMS data set

	DfW *in vivo* CD prediction: 'equivocal' or higher	DfW *in vivo* CD prediction: 'nothing to report'	Totals of experimental calls
Experimental *in vivo* CD positive	4	108	112
Experimental *in vivo* CD negative	0	130	130

Sensitivity: 4/112 = 4%; specificity: 130/130 = 100%; concordance: 134/242 = 55%. CD, chromosome damage.

Figure 4. Example *in vivo* alert in Derek Nexus and information included in the alert.

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test some examples display activity in the liver (Figure 5). For instance, negative results in the mouse BM micronucleus test have been reported for 2,4-dinitrotoluene (1) (11), 2,4,6-trinitrotoluene (2) (11) and 2-amino-4-nitrophenol (3) (12). In contrast, positive results have been observed in the rat liver micronucleus test for 2,4-dinitrotoluene (1) (13), 2,6-dinitrotoluene (4) (13) and 2-nitrofluorene (5) (14) following oral administration.

The mutagenicity and clastogenicity of aromatic nitro compounds is believed to involve reduction by nitroreductases to the hydroxylamine, followed by O-esterification and formation of a reactive nitrenium ion. This reactive species is capable of binding to cellular nucleophiles such as DNA. Considering the observed tissue-specific activity of these compounds, it is possible that one or more of the metabolic steps leading to the formation of the nitrenium ion (Figure 5) requires enzymes present in the liver and that the reactive species generated from metabolic activation is not formed in, nor distributed to, the BM.

Chromium compounds may display route of administration-specific activity

The *in vitro* alert for chromium compounds showed an excellent PP (100%) against *in vivo* data. However, detailed analysis of the experimental data revealed that this activity was dependent on the route of administration used: the identified compounds displayed activity only if administered intraperitoneally and not when given orally (Figure 6). For instance, while positive results in the mouse BM micronucleus test have been reported for lead chromate (6) (15), potassium chromate (7) (16,17) and potassium dichromate (8) (18) following administration by intraperitoneal injection, the latter two compounds failed to induce micronuclei in this tissue when administered orally (16,19,20). The inactivity of chromium (VI) compounds following oral administration has been attributed to poor absorption from the gastrointestinal tract (19).

PAHs with a bay region are more likely to be active in vivo

The *in vitro* alert for polycyclic aromatic hydrocarbons (PAHs) demonstrated a modest PP (50%) against the *in vivo* data. However, closer inspection of the data revealed that a subclass of compounds were mostly active. While bay- and K-region PAHs display activity *in vitro*, data suggest that only bay-region PAHs are active *in vivo* (Figure 7). Examples include, 7,12-dimethylbenz[a]anthracene (21,22), benzo[a]pyrene (9) (23) and dibenz[a,h]anthracene (24). Compounds with a K-region but lacking a bay-region have generally given negative results in *in vivo* cytogenetic tests. For example pyrene (10) (25,26) and phenanthrene (27).

Hydroperoxides may not induce chromosome damage in vivo

For nine of the investigated alerts, the activating compounds were generally negative in *in vivo* cytogenetic tests and it was concluded

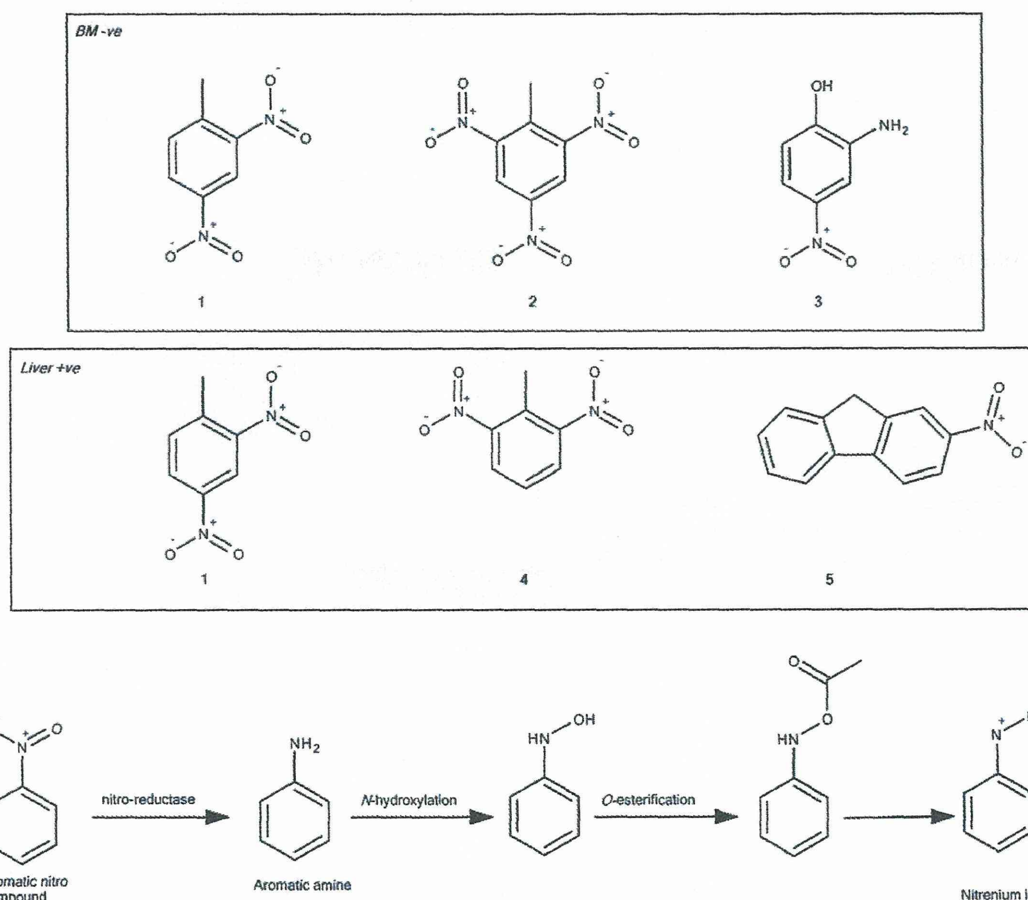

Figure 5. Tissue-specific activity of aromatic nitro compounds and proposed mechanism thought to account for the clastogenicity of these compounds.

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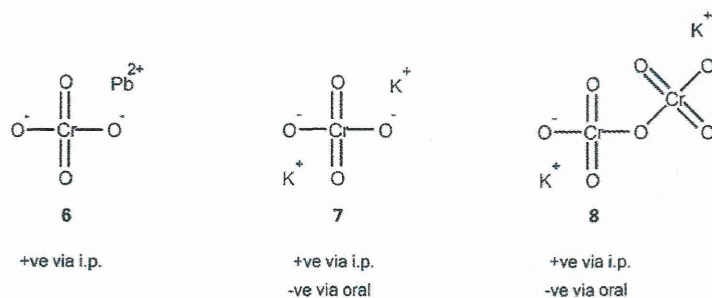

Figure 6. Route of administration-specific activity of chromium compounds.

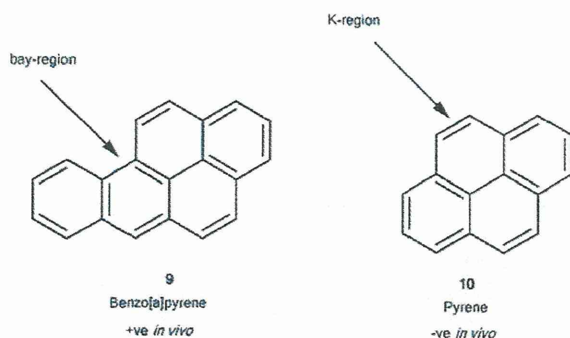

Figure 7. *In vivo* activity of PAHs.

that the *in vitro* alert was not appropriate to describe *in vivo* activity. For these alerts, no new reasoning rule was added, however the alert comments were updated to include a description of the *in vivo* inactivity of the alert class (Figure 3, outcome 4). An example is hydroperoxides. While these compounds show activity in *in vitro* chromosome aberration tests, they do not appear to display activity in *in vivo* assays carried out in BM or peripheral blood. For instance, hydrogen peroxide was reported positive in the *in vitro* chromosome aberration test in the absence of S9 mix (28,29) but negative in the mouse bone marrow micronucleus test, when administered orally and by intraperitoneal injection (30), and in the rat bone marrow chromosome aberration test (31). In these cases it may be that the reactive peroxide compounds administered are adequately deactivated by well developed *in vivo* defence systems in the rodent models. Hydrogen peroxide and other hydroperoxide species can be efficiently metabolised to water/alcohols and oxygen by the peroxidase enzymes (32). These defence mechanisms may not be present or may be overwhelmed in the *in vitro* situation. It should however, be noted that hydrogen peroxide has produced some positive results in carcinogenicity studies. The compound produced carcinoma in the small intestine of mice after oral ingestion in water (33). Therefore, this may again be a case of tissue specific activity although there is not enough *in vivo* genotoxicity assay experimental data to support this hypothesis.

The examples outlined above highlight the richness of information that can be provided with this type of alert and the advantage of having a human expert make an interpretation of the raw data available. They also reflect some more general findings of this work relating to reasons for differences between results observed *in vitro* and those observed *in vivo*. In some cases the discrepancies in results between the *in vivo* and *in vitro* situation can be attributed to various ADME properties. The active compound must reach the tissue being tested and the ability of a chemical species to achieve this may be dependent on a number of factors. If the parent compound causes chromosome damage directly and is reactive, it is important that it reaches the tissue being tested and if this is unclear it should be tested in a tissue where sufficient exposure can be anticipated (usually closer to the site of administration). If a compound is likely to be metabolically activated to the genotoxic species then consideration should be made to whether the *in vivo* system is capable of the required activation. In addition, consideration should also be made of the reactivity of these metabolically activated species with a tissue close to the site of activation being chosen if appropriate (7). In both cases metabolically deactivating pathways present in the *in vivo* systems which may not have been present *in vitro* should also be taken into account.

This type of detailed information was synthesised into a summary for the comments of the alerts updated as part of this work. The findings, interpretation of these findings and any relevant examples are provided along with the relevant references in each case. Figure 8 shows examples of the comments added to the existing alerts described in detail above.

As a result of the fact that all *in vivo* chromosome damage data available to the authors was used to develop the alerts produced in this work (and therefore was effectively within the 'training set' of the model) none of these data would make a good test set by which to validate its performance. However, the broad coverage of chemical space represented by the MMS data set means that this data set provides a good measure of the coverage of the new alerts. At the start of this work there was limited coverage of the endpoint of *in vivo* chromosome damage in the knowledge-based expert prediction system Derek Nexus, as evidenced by the performance statistics of the system against the data set provided by the MMS (Table 1). By combining a large data set of *in vivo* chromosome damage data as well as leveraging knowledge already present in the Derek knowledge base, a significant improvement in the coverage of *in vivo* chromosome damage in Derek Nexus was made in a relatively short time. Against the MMS data set increases in sensitivity and concordance (from 4–40% and 55–61%, respectively) were observed with only a minor concomitant reduction in specificity (a final specificity of 79%; Table 2).

While there is further to go in terms of developing the endpoint (and therefore increasing the sensitivity against this data set) the information available with the alerts currently implemented will already prove very useful to the user when making a decision on the best approach for any further testing required.

Of the 32 *in vitro* chromosome damage alerts investigated during this work, 19 were extended to predict *in vivo* activity, 4 were developed into new *in vivo* SAR alerts, and 9 were considered unsuitable for extension based upon the available data.

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Aromatic Nitro Compound

Excerpt From Alert Comments –

The compounds in this class have generally given negative results in in vivo cytogenetic tests. However, there is limited evidence to indicate that, in some cases, positive results may be observed if the appropriate tissue is chosen for testing. Negative results in the mouse bone marrow micronucleus test have been reported for several aromatic nitro compounds following administration by intraperitoneal injection. Examples include 2,4-dinitrotoluene [Ashby et al], 2,4,6-trinitrotoluene [Ashby et al], 2-amino-4-nitrophenol [NTP 1995] and nitrofurantoin [NTP 1993]. 2-Amino-4-nitrophenol also failed to induce micronuclei when tested in rat bone marrow following administration by oral gavage and intraperitoneal injection [NTP 1994-1995]. In contrast, positive results have been observed in the rat liver micronucleus test for 2-nitrofluorene [Parton and Garriott], 2,4-dinitrotoluene [Takasawa et al] and 2,6-dinitrotoluene [Takasawa et al] following oral administration. These findings suggest that some of the compounds in this class may show tissue-specific activity. While 4-amino-4'-nitrobiphenyl and 4-amino-4'-nitrostilbene have given positive results in the mouse bone marrow chromosome aberration test, it is likely that other functional groups within these structures play a role in their activity [Sinsheimer et al].

Chromium Compound

Excerpt From Alert Comments –

Chromium (VI) compounds have generally given positive results in the in vivo micronucleus test, although studies suggest that the route of administration may play an important role in their activity. For instance, while positive results in the mouse bone marrow micronucleus test have been reported for lead chromate [Watanabe et al], potassium chromate [Wild, Shindo et al] and potassium dichromate [De Flora et al 1990] following administration by intraperitoneal injection, the latter two compounds failed to induce micronuclei in this tissue when administered orally [Shindo et al, De Flora et al 2006, Mirsalis et al]. The inactivity of chromium (VI) compounds following oral administration has been attributed to detoxification in the gastrointestinal tract [De Flora et al 2006]. In 4 week studies, sodium chromate [NTP 1987] and chromium carbonyl [NTP 1993] failed to induce micronuclei in mouse peripheral blood when administered intraperitoneally.

Hydroperoxide

Excerpt From Alert Comments –

The compounds have generally given negative results in in vivo cytogenetic tests. For instance, negative results have been reported for hydrogen peroxide in the mouse bone marrow micronucleus test, when administered orally and by intraperitoneal injection [SCCP], and in the rat bone marrow chromosome aberration test [IARC]. In addition, cumene hydroperoxide [NTP 2004] and methyl ethyl ketone peroxide [NTP 1993] failed to induce micronuclei in mouse peripheral blood in 90 day studies when administered dermally.

Figure 8. Examples of comments added to existing chromosome damage alerts in Derek Nexus as part of this work.

Table 2. Predictive performance of Derek Nexus 2014 KB against the MMS data set

	Derek Nexus *in vivo* CD prediction: 'equivocal' or higher	Derek Nexus *in vivo* CD prediction: 'nothing to report'	Totals of experimental calls
Experimental *in vivo* CD positive	45	67	112
Experimental *in vivo* CD negative	28	102	130

Sensitivity: 45/112 = 40%; specificity: 102/130 = 79%; concordance: 134/242 = 61%. CD, chromosome damage.

Conclusion

The approach described in this work has demonstrated an efficient means of developing a knowledge-based expert prediction system for *in vivo* chromosome damage by leveraging knowledge already captured in a knowledge base relating to compound classes with the potential to cause chromosome damage via specific mechanisms (*in vitro* chromosome damage alerts) in combination with a large data set of *in vivo* chromosome damage data.

The method employed also means that an expert interpretation of the results from the *in vivo* assays could be made before this information was encoded in the predictive system and this interpretation could be taken into account when making the predictions. In addition, as a result of the information provided, the predictions delivered by the *in silico* expert system can be used to direct the user to the most sensitive protocol by which to test these predictions.

The coverage of this endpoint has increased considerably as a result of the work, with the sensitivity against the MMS data set increasing from 4% before its start to 40% at the current time while maintaining a good specificity (79%).

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Limitations in the availability of *in vivo* data activating the remaining alerts may mean that this approach has now reached its limits in terms of increasing sensitivity against this endpoint. However, in the future alternative approaches such as investigating alerts for other related endpoints (e.g. mutagenicity or carcinogenicity) or developing alerts purely based on the *in vivo* data, may help increase coverage of the endpoint further.

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Conflict of interest statement: None declared.

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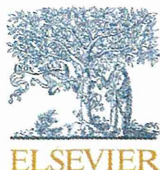

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The JaCVAM international validation study on the *in vivo* comet assay: Selection of test chemicals

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ABSTRACT

The Japanese Center for the Validation of Alternative Methods (JaCVAM) sponsored an international prevalidation and validation study of the *in vivo* rat alkaline pH comet assay. The main objective of the study was to assess the sensitivity and specificity of the assay for correctly identifying genotoxic carcinogens, as compared with the traditional rat liver unscheduled DNA synthesis assay. Based on existing carcinogenicity and genotoxicity data and chemical class information, 90 chemicals were identified as primary candidates for use in the validation study. From these 90 chemicals, 46 secondary candidates and then 40 final chemicals were selected based on a sufficiency of carcinogenic and genotoxic data, differences in chemical class or genotoxic or carcinogenic mode of action (MOA), availability, price, and ease of handling. These 40 chemicals included 19 genotoxic carcinogens, 6 genotoxic non-carcinogens, 7 non-genotoxic carcinogens and 8 non-genotoxic non-carcinogens. "Genotoxicity" was defined as positive in the Ames mutagenicity test or in one of the standard *in vivo* genotoxicity tests (primarily the erythrocyte micronucleus assay). These chemicals covered various chemicals classes, MOAs, and genotoxicity profiles and were considered to be suitable for the purpose of the validation study. General principles of chemical selection for validation studies are discussed.

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1. Introduction

New or modified safety evaluation tests intended for meeting regulatory requirements are evaluated for reliability and relevance by experts, including representatives of regulatory bodies, following a process described in Guidance Document 34 [1] of the Organization for Economic Co-operation and Development (OECD). Evaluating the genotoxicity of chemicals and pharmaceuticals is one of the major safety requirements mandated by various national regulatory agencies. Tests for genotoxicity include both *in vitro* and *in vivo* assays. Generally, although more expensive, *in vivo* assays are considered more relevant than *in vitro* assays in terms of evaluating the potential for human risk. The most common target tissues used for *in vivo* genotoxicity assays are rapidly proliferating cell populations (*e.g.*, hematopoietic cells) that allow the measure of cytogenetic endpoints (*e.g.*, micronuclei). An equally important target organ for evaluating genotoxicity is the liver, the primary site for the metabolic activation of non-reactive chemicals to reactive metabolites. However, this organ consists of terminally differentiated cells that are not readily amenable to evaluating cytogenetic endpoints. Until recently, the primary regulatory assay for detecting genotoxicity in the liver was the unscheduled DNA synthesis (UDS) assay [2]. There are some limitations in the liver UDS assay, which include a comparatively low sensitivity for DNA lesions being repaired by base excision repair [2] and the use of radiolabeled thymidine. Other tests that can target the liver are the liver micronucleus test and the rodent transgenic mutation test; there is currently no OECD guideline for the former test, but recommended

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Table 1
Database of potential test chemicals with genotoxicity and/or carcinogenicity information.

Database	Number of chemicals listed	Note	Refs.
ECVAM list	61	Recommended lists of genotoxic and non-genotoxic chemicals for assessment of the performance of new or improved genotoxicity tests	[9]
IWGT UDS list	131	Overview of published findings from *in vivo* liver UDS tests	[10]
CSGMT list	280	Evaluation of the rodent micronucleus assay in the screening of IARC carcinogens (Groups 1, 2A and B), including results from in vitro genotoxicity tests.	[11]
IARC list	935	Agents reviewed by the IARC monographs, volumes 1–100A, 2 April 2009. Group 1: carcinogenic to humans (108), Group 2A: probably carcinogenic to humans (63), Group 2B: possibly carcinogenic to humans (248), Group 3: not classifiable as to carcinogenicity to humans (515), Group 4: probably not carcinogenic to humans (1).	[12]
CPDB list	1547	Bioassay literature, with qualitative and quantitative analyses of both positive and negative experiments that have been published over the past 50 years in the general literature through 2001 and by the National Cancer Institute/National Toxicology Program through 2004.	[13]
NTP list	ca. 2300	NTP Results reports; Results, status and publication information on all NTP chemicals produced from NTP chemtrack system, 10 August 2000.	[14]
EU GHS list	ca. 4000	Regulation (EC) No 1272/2008 of the European parliament and of the council, List of harmonized classification and labelling of hazardous substances, 31 Decembar 2008. Consideration for carcinogenicity or mutagenicity categories 1A and B, and 2.	[15]

approaches have been made [3–5]. These tests are not used routinely because of their increased complexity, cost, and access to the transgenic animals and/or experimental duration. During the past two decades, increased attention has been directed at the alkaline pH version of the comet assay as an *in vivo* genotoxicity assay [6–8]. All tissues or organs from which single cells can be isolated are potential targets for evaluation, and the assay is relatively easy to conduct and comparatively inexpensive. However, the validation status of this assay had not been formally evaluated. Therefore, it was decided to formally assess the performance of this assay as an *in vivo* alternative to the *in vivo* liver UDS assay. To achieve regulatory acceptance, the reliability and relevance (sensitivity and specificity) of a proposed alternative assay should be comparable to or better than the existing assay currently accepted by regulatory agencies. To evaluate the *in vivo* comet assay, the Japanese Center for the Validation of Alternative Methods (JaCVAM) sponsored a large international prevalidation and validation study. As the primary purpose of genotoxicity tests is the detection of genotoxic carcinogens, both carcinogens and non-carcinogens (genotoxic and non-genotoxic) should be included in any validation study. Herein, we describe the selection process used to identify suitable reference chemicals for the JaCVAM *in vivo* comet assay validation study.

2. Methods

2.1. Chemical selection strategy

The purpose of the JaCVAM sponsored international validation study of the comet assay was to evaluate the ability of the assay to correctly identify genotoxic chemical carcinogens when compared to the currently accepted *in vivo* tests, and additionally to investigate it as an alternative to the rodent liver UDS assay. The reproducibility of the comet assay was also investigated. To achieve this, candidate reference chemicals were selected that belonged to the following four categories: 1) genotoxic carcinogens, 2) genotoxic non-carcinogens, 3) non-genotoxic carcinogens, and 4) non-genotoxic non-carcinogens. The genotoxic information considered was data generated with the rat liver UDS assay and rodent transgenic (TG) mutation assay in liver in addition to the standard tests which included the Ames mutation test, *in vitro* chromosomal aberration test, and *in vivo* rodent erythrocyte micronucleus (MN) test. For the MN test, priority was given to rat data with oral administration rather than mouse data or other routes of administration. Next, secondary candidate chemicals were selected based on factors such as differences in chemical class, structure and mode of action (MOA), ease of handling, availability, budget, *etc.* From this secondary set of candidate chemicals, a final list was selected that would meet the purpose of the validation study.

2.2. Database of potential test chemicals

Several databases that provided appropriate genotoxic and/or carcinogenic information on chemicals were used for primary chemical selection (Table 1); these included:

- ECVAM list

The European Centre for the Validation of Alternative Methods (ECVAM) list of genotoxic and non-genotoxic chemicals for assessing the performance of new or improved genotoxicity tests [9] contains 61 chemicals which are Ames -positive or -negative *in vivo* genotoxins, non-DNA-reactive chemicals (including non-genotoxic carcinogens), and non-carcinogens that are negative or equivocal for genotoxicity *in vivo*.

- IWGT UDS list

The main purpose of the *in vivo* comet assay validation study was to compare the performance of this assay against the *in vivo* rat liver UDS assay for detecting genotoxic chemicals. Therefore, selection of chemicals with liver UDS data was important. The International Workshop on Genotoxicity Testing (IWGT) UDS list is an overview of published findings from *in vivo* liver UDS tests published up to 1993 [10]. The list contains 131 chemicals (36 positives, 81 negatives, 14 equivocal). One hundred and twenty-six chemicals were investigated in male rats, 2 chemicals in female rats, and 20 and 9 chemicals in male and female mice, respectively.

- CSGMT list

The Collaborative Study Group of Micronucleus Test (CSGMT, a working group in the Mammalian Mutagenicity Study group, which is a sub-organization of the Japanese Environmental Mutagen Society) list is the result of a collaborative study on mouse bone marrow and/or peripheral blood micronucleus assays of approximately 100 chemicals classified by the IARC (Groups 1, 2A and B) [11]. The list contains 280 chemicals, which includes 43, 40, and 197 chemicals in Groups 1 (carcinogenic to humans), 2A (probably carcinogenic to humans), and 2B (possibly carcinogenic to humans), respectively, with *in vitro* (Ames test, chromosomal aberration test, and mouse lymphoma tk assay) and *in vivo* (rodent erythrocyte MN or chromosomal aberration test) genotoxicity data.

- IARC list

The IARC list contains agents reviewed by the IARC monographs (volumes 1–100A), as of 2 April 2009 (http://monographs.iarc.fr/ENG/Classification/index.php) [12]. This list includes 935 chemicals/agents, among which 108, 63, 248, 515, and 1 chemicals/agents are in Groups 1, 2A and B, 3 (not classifiable as to carcinogenicity to humans), and 4 (probably not carcinogenic to humans), respectively. IARC monographs provide extensive information including carcinogenic, genetic, and related effects caused by the agents.

- CPDB list

The Carcinogenic Potency Database (CPDB) list is a unique and widely used international resource of the results of 6540 chronic, long-term animal cancer tests on 1547 chemicals (http://potency.berkeley.edu/) [13]. The CPDB provides easy access to the bioassay literature, with qualitative and quantitative analyses of both positive and negative experiments that have been published over the past 50 years in the general literature through to 2001 and by the National Cancer Institute/National Toxicology Program (NTP) through to 2004 and updated in August 2007.

- NTP list

The US NTP list contains results, status, and published information on all chemicals tested by the NTP (http://www.predictive-toxicology.org/data/ntp/original_ntp_data.txt) [14]. It was produced from the NTP chemtrack system dated October 8, 2000. The list contains about 2300 chemicals with data on carcinogenicity, genotoxicity, and organ systems toxicity.

- EU GHS list

The European Union (EU) published a list of harmonised classification and labelling of hazardous substances in Regulation (EC) No 1272/2008 of the European Parliament and of the Council [15]. The globally harmonized system of classification and labeling of chemicals (GHS) is used as the global harmonisation of criteria for classification. The EU GHS list contains about 4000 chemicals which are classified under GHS categories 1A, 1B and 2 for their carcinogenicity or mutagenicity.

2.3. Carcinogenicity and genotoxicity

Information on carcinogenicity (carcinogenic, non-carcinogenic) was based on the IARC evaluations. If the candidate chemicals were not included in the IARC list [12], other databases such as ECVAM [9], CPDB [13], or EU GHS [15] lists were used. As one of the objectives of the comet validation studies is to detect carcinogens in general, rat and/or mouse carcinogens that are considered to act through irrelevant mechanisms for humans or other species were also employed. Information on genotoxicity was based on the lists from ECVAM [9], IWGT UDS [10], CSGMT [11], NTP [14], or IARC monographs [16]. For the chemicals finally selected, precise information on *in vivo* genotoxicity data (mainly rats, but if there were no or only insufficient data in rats, mice were used) was retrieved through a literature survey using PubMed (http://www.ncbi.nlm.nih.gov/pubmed/). In this report, "genotoxic" was defined as positive in the Ames mutagenicity test or in the standard *in vivo* genotoxicity tests (mainly erythrocyte micronucleus assay). A chemical with only positive results in the *in vitro* chromosomal aberration (CA) test was not regarded as genotoxic as the CA test often gave false positive results [9,17,18].

2.4. Chemical properties

Carcinogens are usually divided into genotoxic and non-genotoxic carcinogens. Genotoxic carcinogens included various chemical classes and carcinogenic or genotoxic MOA. These properties were considered during the reference chemical selection process in order to evaluate the applicability domain covered by the *in vivo* rat comet assay. These data were based on the reports from ECVAM [9], CSGMT [11], IARC monographs (http://monographs.iarc.fr/ENG/Monographs/PDFs/index.php) [16], Sasaki et al. [19] or the Hazardous Substance Data Bank (HSDB, http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CHEM).

2.5. Acute toxicity

It was felt important to investigate for acute toxicity data before the start of an experiment as such data can be used to eliminate the need for a dose-finding study or to help set the dose levels to be used in the dose-finding study. Since rats were to be used in the comet assay validation study, rat oral LD_{50} values were investigated using the Registry of Toxic Effects of Chemical Substances (RTECS, http://csi.micromedex.com/fraMain.asp?Mnu=&Restore=Y) or ChemID Plus (http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CHEM).

2.6. Availability

Even if a chemical is considered suitable from the scientific point of view for inclusion as a reference compound in a validation study, there will be cases where it cannot be included due to one or more of the following factors: 1) it is not commercially available or not marketed at sufficient purity, 2) insufficient amounts of chemical in same lot, 3) difficulty in handling of chemicals (gaseous chemical, very small packaging, *etc.*), 4) restriction of transportation to overseas, and 5) high cost. Thus, availability, physical–chemical properties, and price were investigated.

3. Results

3.1. Procedures of the chemical selection

A flow chart describing the different steps involved in the chemical selection process is shown in Fig. 1.

3.2. Primary candidate chemicals (90 chemicals excluding positive control)

The JaCVAM Validation Management Team (VMT) projected that approximately 43 chemicals would be needed in the main comet assay validation study, with approximately 22 genotoxic carcinogens and around 7 chemicals for each of the following categories: genotoxic non-carcinogens, non-genotoxic carcinogens, or non-genotoxic non-carcinogens. The number was based on the need to adequately demonstrate the predictive capability of the assay (sensitivity and specificity) for carcinogenicity, while taking into account the possible number of participating institutions and the anticipated duration of the validation study. Thus, 90 chemicals, excluding a positive control chemical, were selected as primary candidate chemicals (Table 2). This primary candidate list contained about twice the number of chemicals needed in each category.

- Genotoxic carcinogens (43 chemicals)

Forty-three chemicals including three alternatives were selected as candidate genotoxic carcinogens. The three alter-

Table 2
Primary and secondary candidate chemicals for international validation study on the *in vivo* comet assay.

No.	Candidate chemical	CAS	IARC group	CPDB	EU GHS#	Genotoxicity data$				LD50$$	Chemical properties$$$	Price	Note
						Ames	*In vitro* CA	*In vivo*MN##	Liver UDS	Rat, po (mg/kg)	(class, mode of action, *etc*)		
	• Genotoxic* carcinogens (Primary, 43; Secondary, 24; Final, 19)												
1	√2-Acetylamino-fluorene (2-AAF)	53-96-3	NL	+	NL	+	+	+	+	mouse 810	Aromatic amine, metabolic activation, bulky addact	JPY3800/1 g (W)	Used in Phase 4-step 1 study; Selection for inter-laboratory resproducibility
2	Acrylamide	79-06-1	2A	+	Carc. 1B Muta. 1B	−	+	−	−	124	Amide, epoxide	JPY1200/25 g (W)	Non-selection as a final chemical due to use in phase 2 study
3	√ Acrylonitrile	107-13-1	2B	+	Carc. 1B	+	+	+	−	78	Aliphatic cmpd, +ve in rat MN by iv treatment	JPY2100/500 mL (W)	
4	Aflatoxin B1	1162-65-8	1	+	NL	+	+	+	+	2.7	Polycyclic hydrocarbon, metabolic activation, various adducts	JPY45000/5 mL (W)	Non-selection as a secondary candidate due to expensive
5	√*o*-Anisidine (*o*-Anisidine HCl)	90-04-0 (134-29-2)	2B	+	Carc. 1B Muta. 2	+	+	−	−	1150	Aromatic amine, +ve in Ames with norharman, +ve in mouse TG (bladder, but -ve in liver)	JPY1350/25 mL (W)	
6	√ Azidothymidine (AZT)	30516-87-1	2B	+	NL	−	+	+		3084	Heterocyclic cmpd, nucleoside analogue	JPY2500/100 mg (W)	Expesive, but selected
7	Ganciclovir (alternative of azidothymidine)	82410-32-0	NL	NL	NL	−	+	+		mouse > 2000	Heterocyclic cmpd, nucleoside analogue, termination of DNA synthesis		Non-selection as a final chemical due to use of AZT
8	Benomyl	17804-35-2	NL	NL	Muta. 1B	−	+	+		>10 g	Heterocyclic amine, aneugen,	JPY17400/250 mg (S)	Non-selection as a secondary candidate due to no evaluation of carcinogenicity by IARC or CPDB
9	√ Benzene	71-43-2	1	+	Carc. 1A Muta. 1B	−	+	+		930–1800	Aromatic cmpd	JPY780/500 mL(W)	
10	Benzidine	92-87-5	1	+	Carc. 1A	+	+	+	+	309	Aromatic amine	JPY9200/5 mg/mL (1 mL ample) (S)	Non-selection as a secondary caididate due to expensive and unhandy

Table 2 (*Continued*)

No.	Candidate chemical	CAS	IARC group	CPDB	EU GHS[#]	Genotoxicity data[$]				LD$_{50}$[$$]	Chemical properties[$$$]	Price	Note
						Ames	*In vitro* CA	*In vivo*MN[##]	Liver UDS	Rat, po (mg/kg)	(class, mode of action, *etc*)		
11	Benzo[*a*]pyrene	50-32-8	2A	+	Carc. 1B Muta. 1B	+	+	+	–	sc 50	Polycyclic aromatic hydrocarbon, metabolic activation, epoxide, bulky adduct	JPY10000/100 mg (W)	Non-selection as a secondary candidate due to expensive
12	√ Cadmium chloride	10108-64-2	1	+	Carc. 1B Muta. 1B	–	+	+		88	Inorganic metal cmpd, oxidative stress or DNA-repair inhibition?	JPY2500/25 g (W)	
13	Chlorodibromomethane	124-48-1	3	+	NL	+	+	–	–	370	Aliphatic halide	JPY13500/10 g (S)	Non-selection as a secondary candidate due to slimirarity to 1,2-dibromomethane
14	√ *p*-Chloroaniline	106-47-8	2B	–	Carc. 1B	+	+	+		300	Aromatic amine, no adducts	JPY2400/25 g (W)	
15	√ Cisplatin	15663-27-1	2A	NL	NL	+	+	+		25.8	Metal cmpd, cross-linking	JPY123400/5 g (S)	Expensive, but selected.
16	Cyclophosphamide	50-18-0	1	+	NL	+	+	+	–	100	Aziridine, metabolic activation, DNA alkylation	JPY27400/5 g	Non-selection as a secondary candidate due to no stock in the supplier
17	√ 2,4-Diaminotoluene (2,4-DAT)	95-80-7	2B	+	Carc. 1B	+	+	Inc	+	590	Aromatic amime, metabolic activation, weak +ve in rat MN, but -ve in mouse MN	JPY2000/25 g (W)	Used in Phase 2 study; Selection for inter-laboratory reproducibility
18	√ 1,2-Dibromoethane	106-93-4	2A	+	Carc. 1B	+	+	–	+	108	Aliphatic halide	JPY1600/25 mL (W)	
19	3,3'-Dichlorobenzidine	91-94-1	2B	+	Carc. 1B	+		+	+	No data(>1000 mg/kg)	Aromatic amine	JPY12600/25 g (W)	Non-selection as a secondary candidate due to similarity to 2-AAF, *o*-anisidine, and others
20	√ 1,3-Dichloropropene	542-75-6	2B	+	Not classified	+	+	–	–	470	Aliphatic halide	JPY11600/25 g (S)	*in vitro* CA: positive in CHL cells, negative in CHO cells

21	Dichlorvos	62-73-7	2B	+	Not classified	+	+	–	–	17	Aliphatic halide	JPY4000/200 mg (W)	Non-selection as secondary candidates due to expensive
22	3,3′-Dimethoxybenzidine (3,3′-Dimethoxybenzidine 2HCl)	119-90-4 (20325-40-0)	2B	+	Carc. 1B	+	–	+	–	1920	Aromatic amine	JPY5000/25 g (W)	Non-selection as a secondary candidate due to similarity to 2-AAF, *o*-anisidine, and others
23	√ 1,2-Dimethylhydrazine HCl	306-37-6	2B	+	Carc. 1B	+	+	+	+	100	Hydrazine	JPY8800/25 g (T)	
24	7,12-Dimethylbenz[*a*]anthracene	57-97-6	NL	+	NL	+	+	+	–	327	Polycyclic aromatic hydrocarbons, metabolic activation, bulky adduct	JPY35000/1 g (W)	Non-selection as a secondary candidate due to expensive
25	2,4-Dinitrotoluene	121-14-2	2B	+	Carc. 1B Muta. 2	+	–	–	+	268	Cyclic nitro cmpd	JPY1600/25 g (W)	Non-selection as a secondary candidate due to similarity to 2,4- and 2,6-DAT
26	2,6-Dintrotoluene	606-20-2	2B	+	Carc. 1B Muta. 2	+	+	–	+	mouse ca. 700	Cyclic nitro cmpd	JPY5500/25 g (W)	Non-selection as a secondary candidate due to similarity to 2,4- and 2,6-DAT
27	Etoposide	33419-42-0	2A	NL	NL	E	+	+		1784	Heterocyclic cmpd, topoisomerase inhibitor, -ve in TG	JPY10200/25 mg (W)	Non-selection as a secondary candidate due to expensive
28	√ Hydroquinone	123-31-9	3	+	Carc. 2 Muta. 2	–	+	+		302	Aromatic cmpd, aneugen	JPY1300/25 g (W)	
29	IQ	76180-96-6	2A	+	NL	+	+	–		No data	Heterocyclic amine, metabolic activation	JPY31000/100 mg (W)	Non-selection as a secondary candidate due to expensive
30	4,4′-Methylenedianiline (4,4′-Methylenedianiline 2HCl)	101-77-9 (13552-44-8)	2B	+	Carc. 1B Muta. 2	+	+	+	–	No data	Aromatic amine, highest dose of 350 mg/kg in UDS test (rat, po)	JPY2300/25 g (T)	Non-selection as a secondary candidate due to similarity to 2-AAF, *o*-anisidine, and others

Table 2 (*Continued*)

No.	Candidate chemical	CAS	IARC group	CPDB	EU GHS#	Genotoxicity data$				LD50$$	Chemical properties$$$	Price	Note
						Ames	*In vitro* CA	*In vivo* MN##	Liver UDS	Rat, po (mg/kg)	(class, mode of action, *etc*)		
31	√ Methyl methanesulfonate (MMS)	66-27-3	2B	+	NL	+	+	+	+	225	Sulfonate, DNA alkylation	JPY8500/25 g (W)	
32	*N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)	70-25-7	2A	+	Carc. 1B	+	+	+	Inc	90	*N*-nitoroso cmpd, DNA alkylation	JPY8000/5 g (S)	Non-selection as a secondary candidate due to similarity to MMS and MNU
33	*N*-Methyl-*N*-nitrosourea (MNU)	684-93-5	2A	+	NL	+	+	+		110	*N*-nitroso cmpd, DNA alkylation	JPY35800/25 g (S)	Non-selection as a final chemical due to use in phase 3 and phase 4, step 1 studies
34	Mitomycin C (MMC)	50-07-7	2B	+	NL	+	+	+		30	Aziridine, DNA alkylation, DNA cross linker	JPY6800/10 mg (W)	Non-selection as a final chemical due to expensive and to use of busulfan as alternative
35	Daunomycin HCl (alternative of MMC)	23541-50-6	NL	NL	NL	+	+			290	Heterocyclic cmpd, DNA intercalation, inhibition of topoisomerase II, generation of oxygen free radicals		Non-selection as a final chemical due to use of busulfan as alternative
36	√ Busulfan (Myleran) (alternative of MMC)	55-98-1	1	–	NL	+	+	+		mouse 110	Sulfonate, bifunctional alkylating agent (DNA cross-linking)		
37	2-Nitropropane (2-NP)	79-46-9	2B	–	Carc. 1B	+	–	–	+	720	Aliphatic cmpd	JPY1900/25 g (T)	Non-selection as a secondary candidate due to similarity to acrylonitrile
38	*N*-Nitrosodiethylamine	55-18-5	2A	+	NL	+	+	–	+	220	*N*-nitroso cmpd, metabolic activation, DNA alkylation	JPY11300/1 g, JPY21400/10 mL (S)	Non-selection as a secondary candidate due to similarity to DMN
39	√ *N*-Nitrosodimethylamine (DMN)	62-75-9	2A	+	Carc. 1B	+	+	+	+	26	*N*-nitroso cmpd, metabolic activation, DNA alkylation	JPY3400/1 g (W)	
40	√ 4,4'-Oxydianiline	101-80-4	2B	+	Carc. 1B Muta. 1B	+	+	+	–	725	Aromatic amine	JPY2600/25 g (W)	

41	√ Sodium arsenite	7784-46-5	1	–	NL	–	+	+		41	Inorganic metal cmpd, oxidative stress or DNA-repair inhibition?	JPY1900/25 g (W)	
42	Styrene-7,8-oxide	96-09-3	2A	+	Carc. 1B	+	+	–		2000	Epoxide, DNA alkylation	JPY1800/25 mL (W)	Non-selection as a secondary candidate due to similarity to other DNA alkylators
43	√ Thioacetamide	62-55-5	2B	+	Carc. 1B	–	–	+		301	Amide, liver toxicity	JPY2500/25 g (W)	Selection as also liver toxicant
	∘Genotoxic non-carcinogens (Primary, 13; Secondary, 6; Final, 6)												
44	4-Acetylaminofluorene	28322-02-3	NL	–	NL	+	–		–	mouse ip 364	Aromatic amine, analogue of 2-AAF		Non-selection as a secondary candidate due to no suitable supplier
45	√ 9-Aminoacridine (9-Aminoacridine Hydrochloride monohydrate)	90-45-9 (52417-22-8)	NL	NL	NL	+	+			mouse ip 68	Aromatic amine, DNA intercalation	JPY15000/250 g (W)	
46	√ *p*-Anisidine (*p*-Anisidine HCl)	104-94-9 (20265-97-8)	3	–	Not classified	+	+			1320	Aromatic amine, analogue of *o*-anisidine	JPY4600/100g (W)	
47	Methyl chloride	74-87-3	3	NL	Carc. 2	+			+	1800	Aliphatic halide, weak +ve in UDS by inhalation, water soluble gas	JPY3500/0.2 mg/mL (1 mL amplue) (S)	Non-selection as a secondary candidate due to expensive and unhandly
48	√ 2,6-Diaminotoluene (2,6-DAT) (2,6-Diaminotoluene HCl)	823-40-5 (15481-70-6)	NL	–	Muta. 2	+	+	+	Inc	>ca. 300	Aromatic amine, metaqbolic activation, analogue of 2,4-DAT	JPY6500/25 g (W)	Used in Phase 2 study; Selection for inter-laboratory reproducibility
49	√ 5-Fluorouracil (5-FU)	51-21-8	3	+	NL	–	+	+		230	Heterocyclic cmpd, nucleoside analogue, thymidylate synthase inhibitor, +ve only in mice in CPDB	JPY2100/1 g (W)	

Table 2 (*Continued*)

No.	Candidate chemical	CAS	IARC group	CPDB	EU GHS[#]	Genotoxicity data[$]				LD_{50}[$$]	Chemical properties[$$$]	Price	Note
						Ames	*In vitro* CA	*In vivo*MN[##]	Liver UDS	Rat, po (mg/kg)	(class, mode of action, *etc*)		
50	√8-Hydroxyquinoline	148-24-3	3	–	NL	+	+	–	–	1200	Polycyclic hydrocarbon	JPY2450/25 g (W)	
51	4-Nitro-*o*-phenylenediamine	99-56-9	3	–	NL	+	+	–		681	Aromatic amine	JPY3000/25 g (W)	Non-selection as a secondary candidate due to similarity to 2,6-DAT
52	3-Nitropropionic acid	504-88-1	NL	–	NL	+	+			ip 67, mouse po 68	Aliphatic cmpd	JPY56600/10 g (S)	Non-selection as a secondary candidate due to no *in vivo* genotoixcity data
53	6-Mercaptopurine	50-44-2	3	–	NL	+	+	+		277	Polycyclic hydrocarbon, nucleoside analogue	JPY26600/100 g (W)	Non-selection as a secondary candidate due to similarity to 5-FU
54	Phenol	108-95-2	3	–	Muta. 2	–	+	+		317, 512	Aromatic cmpd, +ve in MN might be due to decreasing body temperature	JPY9400/25 g (W)	Non-selection as a secondary candidate due to questionable *in vivo* genotoxicity (herperseramia?)
55	√*p*-Phenylenediamine 2HCl (*p*-Phenylenediamine)	624-18-0 (106-50-3)	3	–	Not classified	+	+	–		147	Aromatic amine	JPY5500/25 g (W)	
56	Thiabendazole	148-79-8	NL	–	Not classified	+	+	+		2080	Polycyclic hydrocarbon	JPY8200/200 mg (W)	Non-selection as a secondary candidate due to expensive
	•Non-genotoxic carcinogens (Primary, 19; Secondary, 7; Final, 7)												
57	Amitrole	61-82-5	3	+	Not classified	–	–	–		1100	Aromatic amine, hormonal effects and prulactin secretion	JPY9000/200 mg (W)	Non-selection as a secondary candidate due to expensive
58	Benzyl acetate	140-11-4	3	+	NL	–	–	–	–	2490	Aromatic cmpd	JPY900/25 g (W)	Non-selection as a secondary candidate due to similarity to di(2-ethylhexyl)phthalate and *o*-phenylphenol Na
59	Chloramphenicol	56-75-7	2A	–	NL	–	+	Inc		2500	Cyclic nitro cmpd, DNA binding	JPY6000/200 mg (W)	Non-selection as a secondary candidate due to expensive

60	√ Chloroform	67-66-3	2B	+	Carc. 2	–	–	–	–	695	Aliphatic halide, liver toxicity	JPY1700/100 mL (W)	Selection as also liver toxicant
61	√ Diethanolamine	111-42-2	2B	NL	NL	–	–	–		ca. 620	Aliphatic cmpd, tumors of mouse liver and renal tubles due to choline deficiency	JPY1350/25 mL (W)	
62	√ Di(2-ethylhexyl)phthalate	117-81-7	2B	+	Not classified	–	–	–	–	30	Aromatic cmpd, peroxisome proliferation	JPY2600/1 g (W)	
63	Diethylstilbestrol	56-53-1	1	+	NL	–	+	Inc		>3000	Aromatic cmpd	JPY8000/200 mg (W)	Non-selection as a secondary candidate due to expensive
64	√ Ethanol	64-17-5	1	+	Not classified	–	–	–		7000	Aliphatic cmpd, liver toxicity	JPY1260/500 mL(W)	
65	Griseofluvin	126-07-8	2B	+	NL	–	+	–		>10 g	Aromatic halide	JPY2700/5 g (W)	Non-selection as a secondary candidate due to no stocks in the supplier
66	Lead acetate	301-04-2	2B	+	Not classified	–	+	Inc		ip 150	Inorganic metal cmpd, vary weak +ve in mice and +ve in rat peripheral blood MN for 10 weeks treatment	JPY5700/25 g (W)	Non-selection as a secondary candidate due to questionable *in vivo* genotoxicity
67	Melamine	108-78-1	3	+	NL	–	–	–		3161	Aromatic amine, bladder and ureteral carcinomas due to calculus formation	JPY6000/100 mg (W)	Non-selection as a secondary candidate due to expensive
68	Methapyrilene HCl	135-23-9	NL	+	NL	E	+		–	200	Aromatic amine		Non-selection as a secondary candidate due to no suitable supplier
69	√ Methyl carbamate	598-55-0	3	+	NL	–	–	–		2500	Amide, inflammation and hyperplasia resulting from bioaccumula-toin, toxic to liver	JPY3700/25 g (W)	Selection as also liver toxicant

Table 2 (*Continued*)

No.	Candidate chemical	CAS	IARC group	CPDB	EU GHS[#]	Genotoxicity data[$]				LD_{50}[$$]	Chemical properties[$$$]	Price	Note
						Ames	*In vitro* CA	*In vivo*MN[##]	Liver UDS	Rat, po (mg/kg)	(class, mode of action, *etc*)		
70	√ *o*-Phenylphenol Na	132-27-4	2B	+	Not classified	–	+	–	–	591	Aromatic cmpd, -ve *in vivo* bone marrow CA test	JPY4400/100 g (W)	
71	Polybrominated biphenyl	67774-32-7	2B	+	NL	–	–	–	–	>1000	Aromatic cmpd	JPY28500/1 mL (W)	Non-selection as a secondary candidate due to expensive
72	Progesterone	57-83-0	2B	NL	NL	–	–			>100	Steroid, carcinogenicity hormonal effects, as progestins in IARC	JPY12500/5 g (W)	Non-selection as a secondary candidate due to no stocks in the supplier
73	√ Saccharin Na	128-44-9	3	+	NL	–	–	–		mouse 17 g	Heterocyclic cmpd, -ve in TG test and adduct, carcinogenicity to male rats only (bladder)	JPY1750/25 g (W)	
74	Terephthalic acid	100-21-0	NL	NL	NL	–	+	–	–	>6400	Aromatic cmpd, rat bladder tumors will be secondary effect due to calculi	JPY1300/25 g (W)	Non-selection as a secondary candidate due to no evaluation of carcinogenicity by IARC or CPDB

75	Trichloroethylene	79-01-6	2A	+	Carc. 1B Muta. 2	–	–	Inc	–	4920	Aliphatic halide	JPY1060/500 mL (W)	Non-selection as a secondary candidate due *to slimilarity to* chloroform
	∘ Non-genotoxic non-carcinogens (Primary, 15; Secondary, 9; Final, 8)												
76	Allyl alcohol	107-18-6	NL	–	Not classified	–		–		64	Aliphatic cmpd, liver tox	JPY2800/25 mL (W)	Non-selection as a secondary candidate due to slimilarity to ethanol
77	√ Ampicillin trihydrate	7177-48-2	3	–	NL	–	–	–		10g	Heterocyclic cmpd, beta-lactam antibiotics	JPY19100/25 g (W)	
78	√ *o*-Anthranilic acid	118-92-3	3	–	NL	–	+	–		5410	Aromatic cmpd	JPY2200/25 g (W)	
79	√ *t*-Butylhydroquinone	1948-33-0	NL	–	NL	–	+	–		700	Aromatic cmpd	JPY2000/25 g (W)	
80	Camptothecine	7689-03-4	NL	NL	NL	–				153	Heterocyclic cmpd, topo I inhibitor	JPY4500/100 mg (W)	Non-selection as a secondary candidate due to expensive
81	Cycloheximide	66-81-9	NL	NL	Muta. 2	–				2	Heterocyclic cmpd, protein synthesis inhibitor	JPY140400/25 g (W)	Non-selection as a secondary candidate due to no evaluation of carcinogenicity by IARC or CPDB
82	√ Ethionamide	536-33-4	3	+	NL	–	+			1320	Amide, carcinogenicity -ve in rats; possible thyroid tumours in mice, +ve only in female mice in CPDB, -ve in both species in other reference, liver toxicity	JPY12500/5 g (W)	Selection as also liver toxicant

Table 2 (*Continued*)

No.	Candidate chemical	CAS	IARC group	CPDB	EU GHS#	Genotoxicity data$				LD$_{50}$$$	Chemical properties$$$	Price	Note
						Ames	*In vitro* CA	*In vivo*MN##	Liver UDS	Rat, po (mg/kg)	(class, mode of action, *etc*)		
83	Eugenol	97-53-0	3	–	NL	–	+	–	–	1930	Aromatic cmpd	JPY8000/200 mg (W)	Non-selection as a secondary candidate due to expensive
84	√Isobutyraldehyde	78-84-2	NL	–	NL	–	+	–		>2000	Aliphatic cmpd	JPY1200/25 mL (W)	
85	D-Mannitol	69-65-8	NL	–	NL	–	–	–		13500	Aliphatic cmpd	JPY1500/25 g (W)	Non-selection as a final chemical due to use in Phase 3 and Phase 4-step 1studies
86	D,L-Menthol	15356-70-4	NL	–	NL	–	+	–		2900	Heterocyclic cmpd	JPY10400/10 g (W)	
87	1-Nitropropane	108-03-2	NL	–	Not classified	–		–	–	455	Aliphatic cmpd, analogue of 2-NP	JPY2500/25 mL (W)	Non-selection as a secondary candidate due to non-selection of 2-NP
88	√Sodium chloride	7647-14-5	NL	–	NL	–	–	–		3000	Inorganic metal cmpd, stomach toxicity	JPY800/500 g (W)	Selection as also stomach toxicant
89	√Trisodium EDTA (monohydrate)	150-38-9 (10378-22-0)	NL	–	NL	–	–			2150	Aliphatic cmpd, chelating agent	JPY2400/50 g (W)	
90	Triton X(-100)	9002-93-1	NL	NL	NL	–				1800	Aromatic cmpd, surfactant	JPY2200/500 mL (W)	Non-selection as a secondary candidate due to no evaluation of carcinogenicity by IARC or CPDB
	• Positive Control (Genotoxic carcinogen)												
91	Ethyl methanesulfonate (EMS)	62-50-0	2B	NL	NL	+	+	+		ip 350,mouse po 470	Sulfonate, DNA alkylation	JPY8000/25 g (W)	

√: Selected as final test chemicals.

*: Genotoxic was defeined as Ames-positive and/or standard *in vivo* assay-positive.

#: EU CLP Regulations (L353/1, 31.12.2008) for carcinogenicity and mutagenicity [15].

##: Erythrocytes (bone marrow or peripheral blood) MN assay with mouse or rat.

$: Data from ECVAM [9], IWGT UDS [10], CSGMT [11], NTP [14] or IARC monographs [16].

$$: Data from Registry of Toxic Effects of Chemical Substances (RTECS) or Chem ID plus.

$$$: Data from ECVAM [9], CSGMT [11], IARC monographs [16], Sasaki et al [19] or Hazardous Substance Data Bank (HSDB).

Abbreviations: CA, chromosomal aberraton; Carc, carcinogenicity; CPDB, carcinogenic potency data base; E, equivocal; GHS, grobally harmonized system of classification and labelling of chemicals; Inc, inconclusive (presence of both positive and negative findings); MN, micronucleus; Muta, mutagenicity; NL, not listed; S, Sigma–Aldrich; T, Tokyo Kasei; TG, rodent transgenic mutation test; UDS, unscheduled DNA synthesis; W, Woko; ip, intraperitoneal injection; iv, intravenous injection; po, per os; sc, subcutaneous injection.

+, Positive.

–, Negative.