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Effects of lowering the proposed top-concentration limit in an *in vitro* chromosomal aberration test on assay sensitivity and on the reduction of the number of false positives

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ABSTRACT

For the *in vitro* chromosomal aberration (CA) test, the proposed top-concentration limit will be reduced to '10 mM or 2 mg/mL' (whichever is lower) in the draft revised OECD (r-OECD) test guideline (TG) 473, down from '10 mM or 5 mg/mL' in the current OECD TG, which was adopted in 1997 (1997-OECD). It was previously reduced to 1 mM or 0.5 mg/mL in the International Conference of Harmonization (ICH) S2 (R1) guideline for pharmaceuticals. Reduction of the top-concentration limit is expected to reduce the number of false or misleading positives. However, this reduction may affect the sensitivity or specificity to predict rodent carcinogenicity. Thus, the effect of a reduction in the top-concentration limit on sensitivity and specificity was investigated by use of a dataset on 435 chemicals obtained from the 'Carcinogenicity and Genotoxicity eXperience' (CGX) database (267 CA-positives and 168 CA-negatives; 317 carcinogens and 118 non-carcinogens) where three TGs (*i.e.*, 1997-OECD, r-OECD and ICH) were applied. The application of the r-OECD TG did not affect the sensitivity (63.1%) or specificity (59.3%) against carcinogenicity, compared with the 1997-OECD TG (sensitivity 63.1%, specificity 59.3%). However, the application of the ICH TG had certain effects, *i.e.*, a decrease in sensitivity (45.4%) and an increase in specificity (72.9%). A change in the number of CA-positives by the application of each TG was also investigated by use of 124 CA-positives from the Japanese Existing Chemical (JEC) database. The application of r-OECD TG showed a small reduction in CA-positives, but the ICH TG reduced this number by approximately half. More than half of the CA-positives had a molecular weight <200. These results suggest that the r-OECD TG will not affect the sensitivity or specificity for the detection of rodent carcinogens, indicating the usefulness of the guideline. However, nearly no improvement with respect to a reduction in the number of false positives should be expected.

1. Introduction

Unless limited by cytotoxicity or solubility, the top concentration suggested for use in the *in vitro* chromosomal aberration (CA) test has been 10 mM or 5 mg/mL (whichever is lower) in the Organization for Economic Co-operation and Development (OECD) test-guideline (TG) number 473 [1] for industrial chemicals and in the International Conference of Harmonization (ICH) S2A guideline [2] for pharmaceuticals, after recommendation from the first International Workshop on Genotoxicity Test Procedures (IWGTP) held in Melbourne in 1993 [3]. The 10-mM limit was defined as a limit that was low enough to avoid arte-factual increases in chromosomal damage due to excessive osmolality and was sufficiently high to ensure the detection of *in vivo* clastogens [4]. However, there has been much discussion on reducing of this top concentration-limit, in particular to diminish the number of false or misleading positive results obtained from mammalian cell genotoxicity tests in recent years [5–10]. Such results are the consequence of biologically non-relevant experimental conditions at very high concentrations used *in vitro*, *e.g.*, low pH, high osmolality, or precipitation of test material in the culture medium. Excessive cellular metabolism, activation or defense, and extremely high concentrations that would not be reached *in vivo* also induce false/misleading positives. Although several recommendations on the new top-concentration limits have been proposed, the recent ICH S2(R1) guideline for pharmaceuticals specified 1 mM or 0.5 mg/mL, whichever is lower, as

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the concentration limit [11]. The rationale for a maximum concentration of 1 mM is as follows: (1) a test battery that includes the Ames test and an *in vivo* genotoxicity assay optimizes the detection of genotoxic carcinogens without relying on any individual assay alone. There is a very low likelihood that the compounds of concern (DNA-damaging carcinogens) – when they are not detected in the Ames test or an *in vivo* genotoxicity assay – can be detected in an *in vitro* mammalian assay above 1 mM; (2) a limit of 1 mM maintains the element of hazard identification, because it is higher than clinical exposures to known pharmaceuticals, including those concentrated in tissues, and is also above the levels generally achieved in preclinical studies *in vivo*. Even beyond the 1-mM limit, the *in vivo* tests ultimately determine the relevance for human safety. However, for pharmaceuticals with an unusually low molecular weight (*e.g.*, less than 200), higher test concentrations should be considered [11]. On the other hand, the draft revised OECD TG 473 proposes a limit of 10 mM or 2 mg/mL, whichever is lower [12]. The rationale for this top-concentration limit is based on the analysis of the data set generated by Parry et al. [6], suggesting that 10 mM is required to detect biologically relevant effects from lower molecular weight non-cytotoxic substances. A simulation study by Brookmire et al. [10] suggested that a test sensitivity at 10 mM is most similar to 2 mg/mL. These findings suggest that the combination of 10 mM or 2 mg/mL, whichever is lower, represents the best balance between the mM and mg/mL concentrations. For complex mixtures, the recommended top concentration remains 5 mg/mL. New top-concentration limits recommended by these TGs are expected to reduce the number of false or misleading positives. However, a reduction of the top-concentration limit may affect the sensitivity or specificity for rodent carcinogenicity, although this reduction should result in an improvement in the specificity of tests without a loss in sensitivity. Here, sensitivity is the ratio of positive *in vitro* CA test results to rodent carcinogens, while specificity is the ratio of negative *in vitro* CA test results to rodent non-carcinogens. In addition, a quantitative structure–activity relationship and software tools have been used recently for to predict genotoxicity [13]. Chromosome damage is also one of the predictive endpoints in in-silico models, *e.g.*, Deductive Estimation of Risk from Existing Knowledge (DEREK) [9,14] or TIssue MEtabolism Simulator (TIMES) [9,15]. Alerts for chromosome damage are based primarily on data from the *in vitro* CA test. Therefore, in-silico evaluation may be affected by changes (from positive to negative) in the CA data. Thus, the effects of reductions of the top-concentration limit on sensitivity and specificity were investigated by use of a set of chemical data, *i.e.*, the Carcinogenicity and Genotoxicity eXperience (CGX) database. To assess the effects in terms of reduction of potential false positives, another chemical data set, *i.e.*, the Japan Existing Chemical (JEC) database, which refers to the Chemical Substances Control Law (CSCL), was used to determine the usefulness of the reduction. These analyses, based on real data obtained for many different chemicals, will be useful for understanding the potential impact of changes in the top concentration used in the *in vitro* CA test.

2. Materials and methods

2.1. Databases used

2.1.1. CGX database

The CGX database provides genotoxicity information on 756 rodent carcinogens and 183 non-carcinogens [16]. The chemicals included in the database comprise all types of chemical, such as industrial chemicals, agrochemicals, pesticides, pharmaceuticals, natural products, and others. For some of these chemicals *in vitro* CA test data are available. The 756 carcinogens included 231 CA-positives, 107 CA-negatives and 14 CA-equivocal. In addition, the 183 non-carcinogens included 61 CA-positives, 61 CA-negatives and 14 CA-equivocal. Data for the *in vitro* CA test were obtained from compilations, such as that from Ishidate et al. [17], and from reports of NTP studies published by Galloway et al. [18], Loveday et al. [19,20], Anderson et al. [21] and other published literature in the CGX database [16]. Thus, various protocols were applied, with different cell types (CHO, CHL, human lymphocytes, etc.), sampling times, top-concentration limit, and cytotoxicity, or different applications of the test guideline or the Good Laboratory Practice (GLP) regulations. The lowest effective concentrations (LECs) were confirmed in all 292 CA-positives (231 carcinogens and 61 non-carcinogens) using the NTP database [22] or original studies [17–21,23–46]. The LEC was defined as the lowest concentration with a statistically significant induction of CA or with a 10% or more CA induction if no statistical analysis was performed, regardless of the test conditions, *e.g.*, different duration of treatment and the presence or absence of S9 mix. The rationale for selecting a 10% cut-off for a positive response is as follows: Ishidate classified test results as positive (≥10% cells with CAs), equivocal (≥5–10% cells with CAs) or negative (less than 5% cells with CAs) in the CA test using Chinese hamster lung (CHL) cells in a similar study protocol [24], and many test results by this author were cited in the CGX database [17]. The 10% cut-off rule is not fully applicable to other cell types with various background data on CA induction in different test protocols. However, it is reasonable to use this cut-off value in order to avoid any overestimation of the CA induction in this analysis. The molecular weight (MW) of each chemical substance was also recorded. When the LEC or MW of the chemical substance could not be identified due to the absence of any description in the paper, *e.g.*, in the case of chemical mixtures or polymers, the substance was excluded from the analysis. Thus, a total of 267 CA-positive chemicals (210 carcinogens and 57 non-carcinogens) were selected for analysis (Table 1). In addition, 168 CA-negatives (107 carcinogens and 61 non-carcinogens) from the CGX database [16] were included. The test concentrations were usually expressed as the weight per volume (*e.g.*, mg/mL). Therefore, LECs identified as mg/mL were converted to equivalent mol concentration (*e.g.*, mM) based on the MW of each chemical.

2.1.2. JEC database

The JEC database, which is based on CSCL regulations, provides toxicity information, *e.g.*, results of a 28-day repeat oral dose study, an Ames test or an *in vitro* CA test, on 277 Japanese existing chemicals (as of January 2012; test data generated from 1991 to 2007) [47]. All chemicals in the database are industrial chemicals with a high production volume in Japan. The *in vitro* CA test was performed with CHL cells according to the OECD TG 473 (first version 1983; revised version 1997 [1]) or the Japanese test guideline for new chemicals [24,48] under GLP conditions. LECs (mg/mL or mM) were defined as those in the CGX database. Of the 272 chemicals with *in vitro* CA data, 124 CA-positives and 148 CA-negatives were found according to their original call (evaluation). Importantly, the old Japanese test guideline employed a long exposure time (48-h of continuous treatment) and the assessment of numerical aberrations for polyploidy was the same as that recently found using TGs. The top-concentration limit was 5 mg/mL (or the equivalent of 10 mM) when no cytotoxicity was observed. The LECs in CA-positives or their MWs were confirmed by use of the original reports [47,49]. All chemicals were identified according to their LECs and MWs; thus, there were no exclusive chemicals identified from the analysis, and 124 CA-positives were used for the analysis (Table 2).

2.2. Application of the test guidelines

The following TGs issued by the OECD and ICH were applied in the analysis: (1) current OECD TG 473 adopted in 1997 (1997-OECD) [1], (2) draft revised OECD TG 473 (r-OECD) [12] for industrial chemicals and 3) ICH S2(R1) TG (ICH) [11] for pharmaceuticals. These TGs specify different top-concentration limits when not limited by solubility or cytotoxicity, namely, 10 mM or 5 mg/mL, whichever is lower, in the 1997-OECD; 10 mM or 2 mg/mL, whichever is lower, in the r-OECD; and 1 mM or 0.5 mg/mL, whichever is lower, in the ICH TG.

2.3. Sensitivity and specificity analyses

To analyze the sensitivity and specificity of the *in vitro* CA-test data against rodent carcinogenicity, a dataset on 435 chemicals (267 CA-positives and 168 CA-negatives; 317 carcinogens and 118 non-carcinogens) from the CGX database was used. Each LEC (in terms of mg/mL and mM) was applied to the three TGs, and the results were re-evaluated (positive or negative) based on the application of the concentration limit for each TG. The sensitivity and specificity against carcinogenicity were also calculated.

2.4. Analysis of the alterations in the number of CA-positives

Analysis of the altered numbers of CA-positives made use of 124 CA-positives from the JEC database. Each LEC (in terms of mg/mL and mM) was applied to the three TGs, and the results (positive or negative) were re-evaluated based on the application of the concentration limit of each TG.

2.5. Evaluation of the relevance of the in vitro CA results

The evaluation of the relevance of the *in vitro* CA results for the chemicals that showed "different" results between the r-OECD (positive call) and ICH (negative call) TGs for chemicals from the JEC database, was based on a weight-of-evidence

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