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Workshop summary: Top concentration for in vitro mammalian cell genotoxicity assays; and report from working group on toxicity measures and top concentration for in vitro cytogenetics assays (chromosome aberrations and micronucleus)

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ABSTRACT

The selection of maximum concentrations for in vitro mammalian cell genotoxicity assays was reviewed at the 5th International Workshop on Genotoxicity Testing (IWGT), 2009. Currently, the top concentration recommended when toxicity is not limiting is 10 mM or 5 mg/ml, whichever is lower. The discussion was whether to reduce the limit, and if so whether the 1 mM limit proposed for human pharmaceuticals was appropriate for testing other chemicals. The consensus was that there was reason to consider reducing the 10 mM limit, and many, but not all, attendees favored a reduction to 1 mM. Several proposals are described here for the concentration limit.

The in vitro cytogenetics expert working group also discussed appropriate measures and level of cytotoxicity. Data were reviewed from a multi-laboratory trial of the in vitro micronucleus (MN) assay with multiple cell types and several types of toxicity measurements. The group agreed on a preference for toxicity measures that take cell proliferation after the beginning of treatment into account (relative increase in cell counts, relative population doubling, cytokinesis block proliferation index or replicative index), and that this applies both to in vitro MN assays and to in vitro chromosome aberration assays. Since relative cell counts (RCC) underestimate toxicity, many group members favored making a recommendation against the use of RCC as a toxicity measure for concentration selection.

All 14 chemicals assayed for MN induction in the multi-laboratory trial were detected without exceeding 50% toxicity by any measure, but some were positive only at concentrations with toxicity quite close to 50%. The expert working group agreed to accept the cytotoxicity range recommended by OECD guideline 487 (55 ± 5% toxicity at the top concentration scored). This also reinforces the original intent of the guidance for the in vitro chromosome aberration assay, where ">50%" was intended to target the range close to 50% toxicity.

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1. Upper concentration limit for in vitro mammalian cell genotoxicity assays

1.1. Introduction

At the 5th International Workshop on Genotoxicity Testing (IWGT) held in Basel, Switzerland in August 2009, one of the major topics under review was the selection of maximum concentrations for in vitro mammalian cell genotoxicity assays. Since the early 1990s, when the OECD guidelines for in vitro mammalian cell genotoxicity assays were developed, considerable experience with these assays has been gained and various analyses of their performance singly and in batteries of tests have been made (e.g., [1–3]). These large retrospective analyses of data bases indicate that the sensitivity of the in vitro mammalian cell genotoxicity tests as currently used is high, but the specificity is low due to positive results for chemicals that are not rodent carcinogens or in vivo genotoxins. Various proposals have been made by expert working groups to reconsider the top concentration used in in vitro mammalian cell genotoxicity assays as a way to increase the accuracy of in vitro testing, with an emphasis on reducing the need for in vivo follow-up testing. Recently, these include recommendations by an ECVAM[3] initiative [4], and in proposed revisions to the ICH S2 guidelines for human pharmaceuticals [5]. Currently, the top concentration recommended for in vitro mammalian cell assays when toxicity is not limiting (e.g., in OECD guidelines) is 10 mM or 5 mg/ml, whichever is lower [6,7]. One topic for the IWGT workshop was to review whether the top concentration limit of 10 mM should be lowered and if so whether a 1 mM limit as proposed in the draft revised ICH S2 guideline for pharmaceuticals [5] was appropriate for testing other types of chemicals. Because this topic was relevant both to the in vitro cytogenetic assays and the mouse lymphoma assay, it was introduced in the first plenary session of the IWGT meeting. The various analyses and proposals on this topic are reviewed below. An account of the discussion of the in vitro cytogenetic assay working group is found in their report below.

The in vitro cytogenetic working group also discussed the appropriate measures and level of cytotoxicity for in vitro cytogenetic assays. A variety of approaches to measurement of cytotoxicity have evolved over the years, which can lead to differences in test agent concentration selection. This topic was addressed in the development of the recent OECD guideline 487 for the in vitro micronucleus (MN) assay, leading to an international trial comparing various measures of cytotoxicity. The initial results of this exercise were reviewed by the IWGT in vitro cytogenetic assays working group who discussed acceptable measures and limits of toxicity. The reports from this OECD trial have since been published [8] and the OECD guideline is now final [9].

2. Upper concentration limit for in vitro mammalian cell genotoxicity assays in the absence of limiting toxicity

2.1. Plenary session. Outline of topics discussed.

2.1.1. ECVAM-sponsored review of maximum concentration in genotoxicty tests of rodent carcinogens

Dr. Raffaella Corvi summarized the results of an evaluation conducted for ECVAM by herself and Drs. James and Elizabeth Parry, in which the published genotoxicity data for mammalian cell assays were examined for 553 rodent carcinogens from the Gold Berkeley Carcinogenic Potency database [10,11]. The objective of the study was to determine how often carcinogenic chemicals gave positive results in selected concentration ranges, e.g., >1–10 mM, ≤1 mM. The authors also examined the Ames test results for chemicals in these various categories to assess the overall results for the chemical in a standard test battery. The summary of this analysis, and an overview of the initial results by Dr. David Kirkland, had been circulated to the expert working groups before the IWGT meeting. The complete report and a follow-up study are now published [10,11]. The key conclusions reported were as follows:

- Of 553 rodent carcinogens in the Gold database, in vitro genotoxicity data that met the study criteria were available for 384. Based on the authors' classification, only 24 carcinogens that were negative in the Ames test were reported as positive in the in vitro cytogenetic assay or mouse lymphoma cell *tk* mutation assay only at concentrations above 1 mM but not exceeding 10 mM (about 6%). Thus, the majority of rodent carcinogens that were positive in the in vitro mammalian cell tests and negative in the Ames test were positive in the in vitro cytogenetic/mouse lymphoma assays at 1 mM or below.[4] The 24 Ames-test-negative rodent carcinogens that were identified as positive only at >1–10 mM in the analysis by Parry et al. [10] were reviewed to assess the weight of evidence available on whether they were likely to have a genotoxic mode of action [11]. Of these 24, it was concluded that 10 chemicals were likely to be carcinogenic by a non-genotoxic mode of action, and 9 potentially had a genotoxic component to their mode of action, or a genotoxic component could not be ruled out. Five carcinogens had an unknown mode of action.
- From the 14 rodent carcinogens that might possibly have a genotoxic component to their mode of action (despite a negative Ames test) and which were genotoxic only at >1–10 mM in mammalian cell assays in vitro, 9 were selected as priorities for re-testing under protocols compliant with current guidelines and standards [11]. One further chemical, diaminozide, was tested because it was reportedly positive at >10 mM, with no data available between 1 and 10 mM. At the time of the IWGT workshop it was already known from some of these re-tests that the original positive reports could not be confirmed; the data for these re-tests, and the review of the "mode of action" data are now published [11]. Of the 10 chemicals that were retested, 5 (benzofuran, monuron, daminozide, 2-mercaptobenzothiazole and toluene) were negative either at the limit of toxicity or at concentrations up to 10 mM. Four of the others (allyl isovalerate, caffeic acid, chlorobenzene and furan) were positive at concentrations <1 mM. Only methylolacrylamide required a higher concentration for detection of a positive response, 2 mM, which, because of the low molecular weight, equated to 202 μg/ml. This re-testing experience demonstrates the improved accuracy of testing when appropriate test protocols are used (a point also emphasized in the mouse lymphoma cell mutation assay working group, elsewhere in this issue).

Overall, it was seen that only a small portion of chemicals that are rodent carcinogens and negative in the Ames test but positive in the in vitro mammalian cell assays are positive only at concentrations above 1 mM, and that many of these rodent carcinogens are likely to operate through a non-genotoxic mode of action. Such

[3] ECVAM is the European Centre for the Validation of Alternative Methods.

[4] In discussion after the meeting, a working group member noted that the actual percentage of chemicals that were negative in the battery would be much greater if the study authors had not selected the lowest concentration reported positive in any mammalian cell assay, (e.g., a result might be positive in the mouse lymphoma assay at < 1 mM, but in the chromosome aberration assay at >1 mM), or accepted positive results in only one type of assay; but the working group member concurred with the general agreement of the workshop, that the majority of rodent carcinogens that were negative in the Ames test were positive in the in vitro mammalian cell assays at 1 mM or below.

chemicals were considered by the authors of these studies likely to represent a low risk of mutagenicity for people under normal exposure conditions.

2.1.2. ICH guideline for testing pharmaceuticals

For human pharmaceuticals, the proposed revisions to the international test guidelines [5] recommend a reduction in top concentration in the in vitro mammalian cell assays to 1 mM, when not limited by solubility or toxicity. Dr. L. Müller briefly reviewed the rationale for this recommendation, which is based on several factors including experience with pharmaceuticals that are uniquely positive in the in vitro mammalian cell assays, where the weight of evidence, based on mechanistic studies in vitro and/or negative results in appropriate in vivo studies, shows that many of the positive results observed at concentrations above 1 mM are not relevant to in vivo conditions and human exposure. The 1 mM limit also takes into account the fact that human exposures to pharmaceuticals, in circulating blood and in tissues, are much lower than 1 mM, even for compounds that accumulate in specific organs. The pharmacologically active concentrations for drugs, and the optimal substrate concentrations for many enzymes including P450s, are typically below 10 μg/ml (20 μM for molecular weight of 500). Data were summarized from information compiled in [12] on human exposure levels for drugs, including those given at high doses, and those known to accumulate in certain tissues. Human peak exposure to pharmaceuticals is generally below 10–50 μM, with exceptions such as some antibiotics, antivirals and antitumor drugs. 17% of 313 marketed pharmaceuticals had a peak exposure (Cmax) over about 50 μM, but all below 1 mM except for the unusual drug lithium (atomic weight 6.9). An important piece of information was that even drugs known to accumulate in tissues to levels 10- to 20-fold higher than plasma levels do not generally reach very high (mM) concentrations in tissues. For example, fluoxetine, a lipophilic drug with a long half-life, has a brain/plasma ratio of 20:1 but accumulates in the brain to ~10 μg/ml (~35 μM), illustrating the finding that known drugs do not have both high plasma levels and 10–20-fold accumulation in tissue.

Typically the molecular weight of pharmaceuticals is relatively high, e.g., 400, so 10 mM represents a concentration in the range of 4 mg/ml. Subsequent to the workshop, an additional notation has been made in note 7 of the ICHS2(R1) guideline, stating: "For pharmaceuticals with unusually low molecular weight (e.g., less than 200) higher test concentrations should be considered."

The current IWGT expert working group considered whether a recommendation to reduce the upper limit for in vitro cytogenetic assays was appropriate for other chemicals (besides human pharmaceuticals), and specifically whether 1 mM was suitable.

2.1.3. Proposal to use a maximum concentration in mg/ml, instead of one based on molarity

An alternative approach to a 1 mM limit was provided to the IWGT group before the meeting by Dr. Bhaskar Gollapudi who was unable to attend. This proposal addressed the concern that a mM limit represented different concentrations for different chemicals when expressed as mg/ml, since molecular weights cover a wide range. [The ability of a chemical to react with DNA is expected to be related to the number of molecules present, i.e., the molarity, so molarity should be the appropriate way to choose test concentrations, but some investigators commonly express concentrations in mg/ml.] Dr. Gollapudi considered that although thinking of test concentrations as a multiple of human exposure might be a practical approach for pharmaceuticals where exposure is known, it was not practical when such human exposure information is not available, and he suggested instead an empirical approach based on the maximum dose recommended by OECD for conducting chronic/subchronic in vivo toxicity studies, a limit dose of 1000 mg/kg/day. If 1000 mg/kg were considered simplistically as equivalent to 1000 mg/l, this in vivo limit concentration could be used as the basis for setting a similar limit concentration for use in vitro of 1000 mg/l, i.e., 1000 μg/ml. The maximum test concentration he proposed was either 10 mM or 1000/MW, whichever is lower. For a chemical with a MW of 25 the concentration tested would be 10 mM, and for one with a MW of 250 the top concentration tested would be 4 mM. This alternative proposal was thus to use a top concentration in vitro of 1000 μg/ml or 10 mM, whichever is lower.

3. Discussion of the upper concentration limit for the in vitro cytogenetics assays

3.1. Report of the in vitro cytogenetic assays working group

In the introduction to the working group session, the history behind the current regulatory guidelines was reviewed. The top concentration recommended for in vitro mammalian cell genotoxicity assays, when toxicity and solubility are not limiting, is 10 mM (e.g. [6]). This recommendation was made, for example, by expert groups developing the OECD guideline and in IWGTP reports [13,14] based on several factors, including: The need to introduce an upper limit to avoid results confounded by high osmolality; the observation that certain mutagens were detectable in the in vitro chromosome aberration assay only at quite high concentrations (between 1 and 10 mM); and a review by Ishidate of lowest effective concentrations in vitro (in an ICPEMC report [15]). In the ICPEMC report, compounds that were positive in vivo in cytogenetic tests were examined to determine what concentrations were required for their detection in vitro in mammalian cell assays, and this was part of the rationale for the 10 mM limit. However, the requirement for 10 mM to obtain positive results viewed the in vitro mammalian cell tests in isolation and not as part of a battery including the Ames test, a component of all test batteries.

3.1.1. Re-examination of the ICPEMC report

A summary statement was made at this IWGT workshop that a re-examination of the data in the report referred to above (Fig. 1 in [15]) shows that of the in vivo positive chemicals, almost all were either detected in the Ames test, or in the in vitro chromosome aberration assay below 1 mM. The exceptions were barbital (which causes kidney tumors in male rats, likely by a non-genotoxic mechanism), and urethane, which is well known as an in vivo genotoxin that is missed by the standard in vitro battery.

3.1.2. Review of in vitro chromosome aberration data in CHL cells

The group also considered a data set from Japan, on results from high production volume chemicals, compiled and presented at the IWGT meeting by Dr. Takeshi Morita. This analysis reviewed in vitro chromosome aberration data that had been generated from 1994 to 2006, in CHL cells. It is recognized that studies prior to 1997 were from protocols that did not follow the OECD guideline published in 1997; the data set was modified to eliminate those that were positive only with a 48 h continuous treatment time that is no longer used. Of 249 chemicals, 45% (113 chemicals) were positive in the in vitro chromosome aberration assay, confirming the high frequency of positive results in this test system. Three compounds were eliminated from analysis because they were positive only at >10 mM, so they would not be detected even by the current 10 mM limit. Of the remaining 110 positive chemicals, 59 were positive for chromosomal aberrations at ≤1 mM and 51 at >1 mM. Of these 51, 14 were positive in the Ames test. Thus, there were 37 chemicals that were positive for chromosome aberrations between 1 and 10 mM that did not give positive results in the Ames test. Of these 37 chemicals, 20 were considered to be of low concern (Table 1).

Table 1
Evaluation of data produced in a Japanese study assessing high volume chemicals for in vitro chromosome aberration induction in CHL cells. Shown are conclusions on the relevance of the responses for 37 chemicals that were positive in the assay at concentrations >1 mM and ≤10 mM, and negative in the Ames test. Evaluation conducted by T. Morita (unpublished).

Relevance	Reason for relevance conclusion	Number	Number negative /number tested in vivo in bone marrow micronucleus assay
"Irrelevant" positive (20)	Low pH	5	2/2
	High toxicity	13	6/6
	In vitro polyploid only	2	1/1
Unknown relevance (17)	Not defined	17[a]	2/2[a]

[a] Eight of these 17 chemicals were positive only with S-9 in the in vitro chromosome aberration assay in CHL cells. Two of these 8 were also negative in the in vivo bone marrow micronucleus assay.

Dr. Morita concluded that the percentage of chemicals that may be "relevant" as a human health risk but are negative in the Ames test and positive in the in vitro chromosome aberration assay only above 1 mM is quite low (17 of the 113 in vitro aberration positive chemicals, or 15%). Further review of the nature of these chemicals is on-going (Morita, in preparation), so the significance of these findings is not known at present.

3.1.3. Discussion of the in vitro cytogenetics working group on the upper concentration limit

The consensus among the working group was that there was evidence supporting the reduction of the upper concentration limit for in vitro cytogenetics assays below 10 mM without significant loss of sensitivity. It was agreed that 1 mM was appropriate for the majority of chemicals in the data sets reviewed, and many members of the group considered that 1 mM could now be recommended as a suitable limit, but this was not a consensus position. One suggestion was that 1–2 mM might be suitable for screening but that higher concentrations might be needed for certain situations. Discussion centered on the possibility of using a 1 mM limit but defining guidance on exceptions, and covered the following points:

a. Inadequate metabolism. It was recognized that some of the chemicals detectable only above 1 mM reflect poor metabolism, for example the requirement for Cyp2E1 which is poorly represented in activation systems such as rat liver S-9 and is needed to activate some chemicals including certain nitrosamines. It was pointed out that inadequate in vitro metabolism should not be a reason to test all chemicals at higher concentrations; it is also known that at high concentrations different metabolic processes may be involved, for example involving P450s that would not normally act on the substrate in question, and producing metabolites that may induce genotoxicity for the wrong reasons; this issue is discussed in more detail in reports of the IWGT [16] and EVCAM [4].
b. Complex mixtures. It was recognized that for complex mixtures a limit expressed in molarity was not practical since molecular weights are not known or are mixed. It was suggested that for complex mixtures, a higher limit such as 5 mg/ml could be maintained, based on an examination by D. Marzin of a set of 20 mixtures; these are addressed in the report of the working group on the mouse lymphoma cell mutation assay in this issue.
c. Impurities. Testing technical grade materials for "qualification" of impurities.
d. Specific chemical classes which exhibit structural alerts for genotoxic carcinogenicity (low molecular weight aldehydes were suggested).
e. Volatility. Test agent volatility is a recognized issue for in vitro assays in general, especially with lower molecular weight chemicals. The group agreed that like metabolism, this illustrates the need for improved test design, not a high concentration limit.
f. High exposure expected (unusually high circulating blood levels, or high exposure at specific localized sites). It was noted that for ethanol, for example, plasma concentrations exceeding 1 mM can occur, and high exposure to certain food ingredients is also known. (Dermal exposure is the subject of a separate concurrent IWGT working group discussion at this workshop [see Pfuhler et al., this issue].)

In discussing how to determine the appropriate conditions for in vitro testing, the overall approach of using rodent carcinogenicity assays as the sole standard against which the performance of short term tests for genetic toxicity is measured was questioned by some workgroup members. Because of the very brief time available, the group agreed that the topic of how to determine suitable top concentrations required further discussion.

4. Final plenary session: conclusions on the upper concentration limit

In the final follow-up plenary session, there was further discussion of upper concentration limits for in vitro mammalian cell assays on chemicals other than human pharmaceuticals. There was consensus that there was reason to consider reducing the 10 mM limit, and many attendees favored a reduction to 1 mM. However, there was not agreement on what the upper concentration limit should be. Several alternative proposals were put forward. The first listed below, (a), had been discussed by the in vitro cytogenetics working group, but the others had not been discussed in detail to determine whether the same exceptions would apply.

a. 1 mM may be acceptable for routine testing
   Exceptions proposed included low molecular weight substances, certain chemicals with high (particularly local) exposure, complex mixtures, and technical grade materials tested to assess impurities.
b. 2 mM may be acceptable for routine testing
   Similar exceptions to above may apply
c. 1 mM or 500 μg/ml, whichever is the higher, may be acceptable
d. A top concentration of 1000 μg/ml or 10 mM, whichever is the lower, may be acceptable
   In practice, this would mean that low MW chemicals would be tested to 10 mM but many chemicals would be tested to about 2–4 mM.

5. Appropriate measures and level of cytotoxicity for in vitro cytogenetic assays

5.1. Report of the in vitro cytogenetic assays working group

In vitro cytogenetic assays have been used for decades. In early testing, concentrations were often limited only by the ability to recover sufficient cells to score. Since then several approaches to the measurement of cytotoxicity have evolved, often with more precise analyses that take into account cell proliferation/growth. Differences in the way in which toxicity is measured lead to differences in concentration selection and assay acceptability, and have been discussed extensively [17–21]. This topic arose during the development of the recent OECD guideline for the in vitro MN

Table 2
Common measures of cytotoxicity used for in vitro cytogenetic assays.

Measure (defined below)	Abbreviation (cf control)	Chromosome aberration assay	Micronucleus assay	Measure provides indication of proliferation
Mitotic index	RMI (relative mitotic index)	√		Yes
Binucleate cell index with cytochalasin B/replicative index	CBPI/RI (cytokinesis block proliferation index/replicative index)		√	Yes
Population doubling (without cytochalsin B in micronucleus assay)	RPD (relative population doubling)	√	√	Yes
Increase in cell counts (without cytochalsin B in micronucleus assay)	RICC (relative increase in cell count)	√	√	Yes
Cell counts (without cytochalsin B in micronucleus assay)	RCC (relative cell count)	√	√	No

Definitions: calculations of toxicity measures (usually expressed as percentages):

RMI	[No. mitotic cells/total No. cells (*treated*)]/[No. mitotic cells/total No. cells (*control*)]
CBPI	([(No. mononucleate cells) + (2 × No. binucleate cells) + (3 × No. multinucleate cells)])/(Total No. cells)
RI	([(No. binucleate cells) + (2 × No. multinucleate cells) cells)]/(Total No. cells) *treated*)/([(No. binucleate cells) + (2 × No. multinucleate cells)]/(Total No. cells) *control*)
RICC	[Final No. cells – initial No. cells (*treated*)]/[Final No. cells – initial No. cells (*control*)]
RPD[a]	(No. of Population doublings *treated*)/(No. of population doublings *control*)

No., number.
[a] Population Doubling = [log(final cell number/initial cell number)]/log 2.

assay [9], leading to a multi-laboratory, international trial to compare various approaches for measuring cytotoxicity. The results of this study were reviewed at the IWGT meeting and have since been published (summarized in [8,22]); there was overall agreement on using measurements based on cell proliferation for cytogenetic assays. The results of the IWGT working group discussions are reported below. For the purposes of this meeting, cytogenetic assays were defined as both types of in vitro chromosome damage assays: chromosome aberration assays where cells are analyzed at metaphase, and MN assays conducted either with or without Cytochalasin B (CytoB).

5.1.1. Review of measures of cytotoxicity

It is recognized that a balance is required in choosing toxicity limits to obtain appropriate sensitivity and specificity of in vitro mammalian cell testing. To help ensure sensitivity, high concentrations of a test article are used, but excessive toxicity can result in "false" negative results if cell growth is delayed so that they do not reach metaphase, or they fail to progress through metaphase into the next interphase where micronuclei can be detected. On the other hand, excessive toxicity can give rise to "misleading" positive results that occur only under cytotoxic conditions and not at lower concentrations. They are considered misleading because the events that lead to chromosome aberrations originate directly or indirectly from the processes that result in toxicity and that do not operate at lower concentrations relevant to normal usage or to human exposure; thus the dose response is expected to be non-linear.

The measures of cytotoxicity that are commonly used (Table 2) were reviewed by the in vitro cytogenetic working group. As indicated in Table 2, the relative cell count (RCC) is a measure of cytotoxicity that does not provide information on cell proliferation. An example is provided to demonstrate how RCC can underestimate toxicity (Table 3). (For simplicity, this example shows results after only one doubling in the controls.) In this example, cell numbers in the treated population did not increase during the assay (either because there was no cell proliferation, or cell proliferation was balanced by cell death), whereas the control population doubled in cell number between the beginning of treatment and the harvest, as expected. The calculation of relative population doubling (RPD) and relative increase in cell count (RICC), in this case zero, reflects the fact that there is no increase in the population of cells, i.e. the treated culture experiences 100% toxicity relative to the increase observed in the control culture. The calculation of RCC, which is based on comparing the cell counts only at the end of the assay, indicates 50% fewer cells in the treated culture compared to the control, or only 50% toxicity. In this example, toxicity is greatly underestimated when calculated by RCC compared to RPD and RICC.

Table 3
An example comparing the effects of using RPD/RICC or RCC to estimate cytotoxicity.

Culture	Initial cell number at treatment start (million)	Final cell number (million)	% toxicity (by RPD or RICC)	% toxicity (by RCC)
Control	0.5	1.0		
Treated	0.5	0.5	100% (no growth)	50% (50%)

Because micronuclei can only be observed in cells that have gone through mitosis, the expert working group affirmed the conclusion reached previously by various investigators and expert groups [18–21,23,24], i.e., that toxicity measures that provide information on the amount of cell proliferation that occurs after the beginning of treatment are preferred for measuring toxicity in cytogenetic assays.

5.1.1.1. Review of data generated using different measures of cytotoxicity. Data were reviewed from collaborative studies conducted on 14 chemicals in 12 laboratories as part of the process for drafting OECD guideline 487 for the in vitro MN assay. The results were summarized at the IWGT meeting by Kirkland and Schuler, and details for some of the chemicals were presented. The data are in individual reports in a special issue of *Mutation Research*, with a summary and conclusions by Kirkland [8,22]. The conclusions are summarized here.

These studies assessed:

- Multiple toxicity measures concurrently
- Five cell lines: L5178Y mouse lymphoma cells; TK6 human lymphoblastoid cells; and three hamster cell lines, CHO, CHL and V79.
- 14 clastogens and aneugens of various chemical classes/mechanisms, including strong and weak genotoxins: aminoanthracene, benzo[*a*]pyrene; cadmium chloride; colchicine; cyclophosphamide; cytosine arabinoside; diazepam; diethylstilboestrol; etoposide; 5-fluorouracil; mitomycin C; phenolphthalein; quinacrine and vinblastine.

The studies followed the draft OECD guideline 487 for the in vitro MN assay and each chemical was tested in at least 2 laboratories and in as many cell types as possible. (The OECD guideline has since been published as a final guideline [9].) Not every chemical was tested in every cell type. Every laboratory conducted tests in the absence of CytoB, but some laboratories also conducted tests in the presence of CytoB so that the cytokinesis block proliferation index [CBPI] and replication index [RI] could be compared with measures used for assays conducted in the absence of CytoB (i.e., RCC, RICC, and RPD). For all of the chemicals, the method used for estimating toxicity affected the concentrations selected for scoring. For all chemicals and for each cell type, either the extent of toxicity according to RCC at a given concentration was less than that according to RICC, or the concentration required to achieve a particular level of cytotoxicity was higher in the case of RCC than for RICC. Thus RICC never identified a higher concentration target range for testing than did RCC.

The results indicate that all of the measures of cytotoxicity (RICC, RPD, CBPI/RI and RCC) allowed detection of all the chemicals (both weak and potent chemicals) at or below an upper limit of 55 ± 5% toxicity, as recommended in OECD guideline 487. In the presence of CytoB there was one exception, quinacrine, which did not give a significant increase in micronuclei with 56% suppression of proliferation (CBPI); this may be related to the well known very narrow active concentration range for detecting positive responses with aneugens (see for example [25]). Increases in micronuclei were seen with quinacrine when cytoB was not used [26].

It was noted that good study design may require closely spaced concentrations (much closer than half-log or doubling dilutions) particularly for aneugens, some nucleoside analogues and other test agents with steep toxicity dose relationships.

The use of CytoB was also discussed briefly. CytoB prevents cell division following nuclear division, resulting in binucleated cells. Thus micronuclei can be scored in interphase cells that are clearly identified as having recently divided. In some cases however, micronuclei are observed in mono-nucleated cells in preparations treated with CytoB, and the results of the collaborative study on 14 chemicals (above) reaffirmed that when increases in micronuclei are concurrently noted in mononucleated cells, they should be scored (a; see below). It was also noted that based on experience with compounds that interfere with mitosis, when cells with asymmetrical multinucleated morphology are seen (b; see below), they can complicate scoring and indicate the need to repeat the assay without CytoB (see for example [26]). Asymmetrical multinucleated morphology has been seen for example with phenolphthalein, and with "reference" aneugens such as taxol, colchicine and vincristine.

The group recommended that if equivocal results are found in an in vitro MN assay in either of these two situations (a or b in the preceding paragraph), a repeat test without CytoB is needed. It was noted by M. Schuler that in certain circumstances CytoB can increase the toxicity of a test chemical, examples being diazepam, or the increase in apoptosis with CytoB and phenolphthalein.

5.1.1.2. Conclusions on measures of cytotoxicity. Taking into consideration the principle illustrated in Table 3, and the results of the comparison of the various measures of cytotoxicity in the collaborative MN trial, the expert working group agreed on a preference for using methods for measuring toxicity that take cell proliferation after the beginning of treatment into account (RICC, RPD, CBPI/RI). Further, since RCC underestimates toxicity, many group members favored making a recommendation against the use of RCC as a toxicity measure for concentration selection. This was not a consensus position however, and it was noted that an ongoing study (Fowler et al., in preparation) will provide further information on RCC. (RCC is not one of the recommended toxicity measures in the OECD guideline for in vitro MN testing [9].) This data collection and review by Fowler and others is an examination of chemicals that give "misleading positive" results (Ames test-negative chemicals that are positive in vitro in mammalian cell assays but are negative in standard in vivo genotoxicity assays or carcinogenicity assays, or induce tumors via an accepted non-genotoxic mode of action e.g., sodium saccharin).

The group also agreed that the conclusion on the preference for a toxicity measure that indicates cell proliferation following the beginning of treatment applies both to in vitro MN assays and to in vitro chromosome aberration assays (where mitotic index is an additional measure, for assays in lymphocytes).

5.1.2. Review of upper limit of cytotoxicity

In the early 1990s expert groups developing the OECD guideline 473 [6], and the IWGTP group [13,14] recommended an upper limit of 50% cytotoxicity for the in vitro chromosome aberration assay. However, the final recommendation in the guideline was ">" 50%, a change that was made for practical purposes to avoid repeated attempts to obtain exactly 50% cytotoxicity. This has unintentionally led to the frequent testing of excessively toxic concentrations. International discussions of a suitable upper limit of toxicity for the in vitro MN assay have led to the recommendation in OECD guideline 487 [9] that the upper limit should be 55 ± 5% toxicity at the top concentration scored. At the current IWGT meeting, it was noted that all 14 chemicals assayed for MN induction in the recent interlaboratory trial were detected without exceeding 50% toxicity by any measure, but some chemicals were positive only at concentrations with toxicity quite close to 50% b [8,22].

5.1.2.1. Conclusions on upper limit of toxicity. The expert working group agreed to accept the cytotoxicity range recommended by OECD guideline 487 [9], namely to achieve 55 ± 5% toxicity at the top concentration scored. This also reinforces the original intent of the guidance for the in vitro chromosome aberration assay, where >50% was intended to target the range close to 50% toxicity.

Conflict of interest

None declared.

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ORIGINAL PAPER

Chiral analyses of dextromethorphan/levomethorphan and their metabolites in rat and human samples using LC-MS/MS

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Abstract In order to develop an analytical method for the discrimination of dextromethorphan (an antitussive medicine) from its enantiomer, levomethorphan (a narcotic) in biological samples, chiral analyses of these drugs and their *O*-demethyl and/or *N*-demethyl metabolites in rat plasma, urine, and hair were carried out using LC-MS/MS. After the i.p. administration of dextromethorphan or levomethorphan to pigmented hairy male DA rats (5 mg/kg/day, 10 days), the parent compounds and their three metabolites in plasma, urine and hair were determined using LC-MS/MS. Complete chiral separation was achieved in 12 min on a Chiral CD-Ph column in 0.1% formic acid–acetonitrile by a linear gradient program. Most of the metabolites were detected as being the corresponding *O*-demethyl and *N*, *O*-didemethyl metabolites in the rat plasma and urine after the hydrolysis of *O*-glucuronides, although obvious differences in the amounts of these metabolites were found between the dextro and levo forms. No racemation was observed through *O*- and/or *N*-demethylation. In the rat hair samples collected 4 weeks after the first administration, those differences were more clearly detected and the concentrations of the parent compounds, their *O*-demethyl, *N*-demethyl, and *N*, *O*-didemethyl metabolites were 63.4, 2.7, 25.1, and 0.7 ng/mg for the dextro forms and 24.5, 24.6, 2.6, and 0.5 ng/mg for the levo forms, respectively. In order to fully investigate the differences of their metabolic properties between dextromethorphan and levomethorphan, DA rat and human liver microsomes were studied. The results suggested that there might be an enantioselective metabolism of levomethorphan, especially with regard to the *O*-demethylation, not only in DA rat but human liver microsomes as well. The proposed chiral analyses might be applied to human samples and could be useful for discriminating dextromethorphan use from levomethorphan use in the field of forensic toxicology, although further studies should be carried out using authentic human samples.

Keywords Levomethorphan · Dextromethorphan · Chiral analysis · Biological samples · LC-MS/MS · Enantioselective metabolism

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Introduction

Dextromethorphan is widely used all over the world as an over-the-counter antitussive medicine. It produces little or no central nervous system depression at therapeutic doses, but it has dissociative effects similar to ketamine and phencyclidine in large doses as an *N*-methyl-*D*-aspartate receptor antagonist [1–6]. To obtain its hallucinogenic effect, young people abuse this drug by large doses and many fatalities from overdoses have been reported [7, 8]. In

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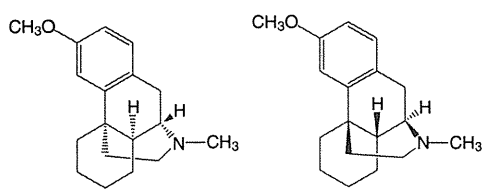
contrast, its enantiomer, levomethorphan, is a potent narcotic analgesic [9] (Fig. 1), and an *O*-demethyl compound of levomethorphan, levorphanol, is known to have stronger opioid pharmacological effects [9, 10]. Levomethorphan is strictly controlled in the world as a narcotic and is never used for therapeutic purposes.

In humans, as shown in Fig. 2, it has been reported that dextromethorphan is primarily metabolized to dextrorphan via *O*-demethylation by cytochrome P450 2D6 (CYP2D6), which is polymorphically expressed in humans, who can be classified as poor, intermediate, and extensive metabolizers [11, 12]. Dextromethorphan is *N*-demethylated via an additional route to 3-methoxymorphinan (3-MEM), primarily mediated by CYP3A4 in human liver microsomes [11, 13]. Dextrorphan and 3-MEM are then demethylated to 3-hydroxymorphinan (3-HM) via CYP3A4 and CYP2D6, respectively. Moreover, dextrorphan and 3-HM are glucuronized to their *O*-glucuronides and these are mainly excreted into human urine [14, 15].

A variety of analytical methods for the determination of dextromethorphan and its metabolites in biological samples have been reported using capillary electrophoresis (CE) [16, 17], HPLC [18–22], GC-MS [23–26], and LC-MS (/MS) [15, 27–30]. However, there is little information regarding the metabolic properties of levomethorphan. Although a chiral separation method of dextromethorphan and levomethorphan using HPLC with fluorometric detection [22] or using CE [17] has been reported, there has been no report that describes a simultaneous determination of dextromethorphan, levomethorphan, and their metabolites in biological samples after administration of these drugs. Considering the possibility of the adulteration or substitution of dextromethorphan with levomethorphan due to its chemical similarities for illegal purposes, it is necessary to establish the enantiometric separation of dextromethorphan and levomethorphan in biological samples.

In order to develop an analytical method for the discrimination of dextromethorphan from levomethorphan in biological samples, we first investigated chiral analyses of these drugs and their *O*-demethyl and/or *N*-demethyl metabolites in plasma, urine, and hair samples of rats administered with each enantiomer, using LC-MS/MS. In addition, detailed metabolic properties of these drugs were investigated using rat and human liver microsomes.



Dextromethorphan Levomethorphan

Fig. 1 Chemical structures of dextromethorphan and its enantiomer, levomethorphan

Experimental

Materials

Levomethorphan was obtained from Cerilliant (Round Rock, TX, USA). Dextromethorphan hydrobromide, dextrorphan tartrate, (+)-3-HM hydrobromide, (+)-3-MEM hydrochloride, and levallorphan tartrate (used as internal standard, IS) were from Sigma-Aldrich (St. Louis, MO, USA). Levorphanol tartrate was given by Professor T. Nagano (Graduate School of Pharmaceutical Sciences, The University of Tokyo, Japan). A β-glucuronidase solution (EC 3.2.1.31, 103,000 units/mL, Source: *Helix pomatia*) was purchased from Wako Chemicals (Osaka, Japan). A solid-phase extraction column (OASIS HLB, 3 cc, 60 mg) was obtained from Waters (Milford, MA, USA), and the membrane filter (Ultrafree-MC, 0.45 μm) was from Millipore Corporation (Bedford, MA, USA).

Liver microsomes from individual male dark agouti (DA) rats (n=4, 6 weeks old, around 125 g mean weight) were prepared by ultracentrifugation as described [31, 32]. The concentrations of microsomal protein were estimated using a bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL). Human liver microsomes (50-donor pool, 20 mg/mL) were purchased from BD Biosciences (Woburn, MA, USA). Nicotinamide adenine dinucleotide phosphate (NADP) and glucose 6-phosphate (G-6-P) were obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan) and G-6-P dehydrogenase (G-6-PDH) was from Roche Diagnostics (Indianapolis, IN, USA). All other chemicals and solvents were of an analytical reagent grade or HPLC grade (Wako Chemicals, Osaka, Japan).

Instrumentation

The UPLC analysis was performed using a Waters Acquity Ultra-Performance™ liquid chromatography system (Waters, Milford, MA, USA). The separations were achieved using a Chiral CD-Ph column (150×2.1 mm i.d., 5 μm) from Shiseido (Tokyo, Japan). The column temperature was maintained at 30 °C, and the following gradient system was used with a mobile phase A (0.1% formic acid) and mobile phase B (0.1% formic acid/acetonitrile) delivered at 0.25 mL/min: 80% A/20% B (2 min hold)—70% A/30% B (15 min). The mobile phase was used as a wash solvent to avoid any carry-over from previous injections. The autosampler was maintained at 4 °C, and the injection volume was 2 μL. Quantitation was achieved by MS/MS detection in a positive ion mode using a Quattro Premier XE mass

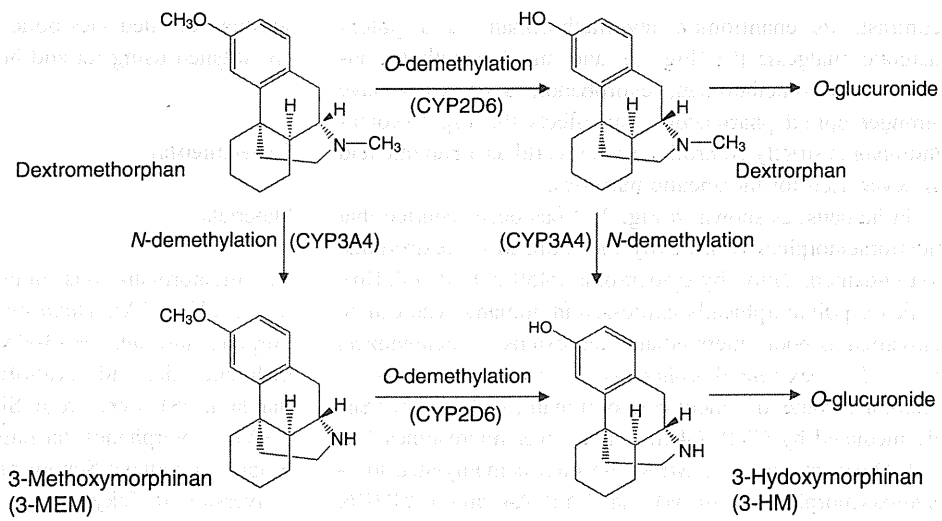


Fig. 2 Main metabolic pathway of dextromethorphan in humans

spectrometer (Waters, Milford, MA, USA) equipped with an electrospray ionization (ESI) interface. Quantification was performed using multiple reaction monitoring of the transitions of precursor ions to product ions with each cone voltage and collision energy as shown in Table 1. The optimal MS parameters obtained were as follows: capillary 3.0 kV, source temperature 120 °C, and desolvation temperature 350 °C. Nitrogen was used as the desolvation and cone gas, with a flow rate of 800 and 50 L/h, respectively. Argon was used as the collision gas, with a flow rate of 0.25 mL/min. All data collected in the centroid mode were processed using MassLynx™ NT4.1 software with a QuanLynx™ program (Waters, Milford, MA, USA).

Since the standard compounds of (−)-3-MEM and (−)-3-HM were not available, these peaks were confirmed by comparison of their retention times and mass fragmentations with those of the standard compounds of the dextro forms ((+)-3-MEM and (+)-3-HM) using an ODS column. The analyses were performed using an Acquity HSS T3 column (100×2.1 mm i.d., 1.8 μm) from Waters (Milford, MA, USA). The column temperature was maintained at 40 °C, and the following gradient system was used with a mobile phase A (1% formic acid) and mobile phase B (1% formic acid/acetonitrile) delivered at 0.3 mL/min: 90% A/10% B (0 min)–70% A/30% B (8 min). The MS parameters were the same as for the analyses using the chiral column described above.

Animal experiments

The animal experimental model was designed as shown in our previous reports [33, 34]. All experiments were carried out with the approval of the Committee for Animal Care and Use of the National Institute of Health Sciences, Japan. Dextromethorphan hydrobromide (dissolved in an isotonic sodium chloride solution, 2.5 mg/mL, rat 1–3) or levomethorphan (dissolved in a mixed solution of 5% Emulphor™ EL-620/5% ethanol/90% isotonic sodium chloride solution, 2.5 mg/mL, rat 4–6) was administered to male DA pigmented rats, which were 5 weeks old and around 90 g mean weight (Japan SLC, Shizuoka, Japan). The drugs were given once daily at 5 mg/kg by intraperitoneal injection for ten successive days. Blood samples were collected 5, 15, 30, 60, 120, and 360 min after the first administration from the orbital vein plexus. Plasma samples were prepared by centrifugation at 10,000×*g* for 3 min and stored at −20 °C until analysis. The area under the plasma concentration time curve (AUC) was calculated by the conventional method. Urine samples were collected 0–24, 24–48, and 48–72 h after the last administration and stored at −20 °C. Each animal had been shaved on the back just before the first drug administration. The new growing hair samples were collected 28 days after the first administration.

Extraction of parent compounds and their metabolites from rat plasma and urine samples

For the quantitative analysis of *O*-demethyl and *N*,*O*-didemethyl metabolites in the rat plasma and urine samples, the analytes were measured as free compounds after the hydrolysis of *O*-glucuronides. The optimal condition of the hydrolysis was evaluated, with the peak of putative *O*-glucuronide at nearly 2 min (*m/z* 434→258) on the MRM chromatogram disappearing from rat plasma and urine samples after the hydrolysis.

A 25-μL plasma sample with 50 μL of added 10 mM ammonium formate buffer (pH 5.0) was reacted with 20 μL of the β-glucuronidase solution at 37 °C for 20 h. To precipitate the proteins in the plasma, 40 μL of the IS methanol solution and 100 μL of methanol were poured into each tube, and the mixtures were then vigorously mixed. The

Table 1 Analytical conditions of LC-MS/MS using the Chiral CD-Ph column

Compounds	Retention time min	Precursor ions *m/z*	Cone voltage V	Product ions *m/z*	Collision energy eV
Dextromethorphan	10.6	272	40	171	45
Dextrorphan	6.1	258	45	157	40
(+)-3-MEM	8.1	258	40	170	35
(+)-3-HM	3.9	244	30	156	35
Levomethorphan	11.3	272	40	171	45
Levorphanol	5.5	258	45	157	40
(−)-3-MEM	9.8	258	40	170	35
(−)-3-HM	4.5	244	30	156	35
Levallorphan (IS)	7.5	284	40	157	40

mixed solution was centrifuged at 1,200×*g* for 3 min and filtered prior to the injection for the LC-MS/MS analysis.

To a 50-μL urine sample (20 μL for 0–24 h samples) was added 100 μL of the β-glucuronidase solution, 1 mL of 10 mM ammonium formate buffer (pH 5.0) and 50 μL of the IS aqueous solution, respectively. The mixed solution was incubated at 37 °C with gentle shaking. After an OASIS HLB column was pre-activated with 2 mL of methanol and distilled water, the reaction mixture was applied to the column. Following the wash of the column with 2 mL of distilled water, 1 mL of methanol was passed through the column to elute the target drugs. A 2-μL of the solution was automatically injected into the UPLC-MS/MS.

Extraction of parent compounds and their metabolites from rat hair samples

Hair samples were washed three times with 0.1% sodium dodecyl sulfate under ultrasonication, followed by washing three times with water under the same condition. After the sample was dried under a nitrogen stream at room temperature, approximately 10 mg of finely cut hair was precisely weighed and extracted with 1 mL of methanol/5 M hydrochloric acid mixed solution (20:1) containing 50 μL of the IS methanol solution for 1 h under ultrasonication. Following overnight storage at room temperature, the hair was filtered off, the filtrate was evaporated with a nitrogen stream, and the residue was dissolved in 1 mL of distilled water. The solution was treated with an OASIS HLB column and analyzed as described above.

Linearity, precision, and recovery of the analytical method for the rat samples

An individual standard solution of 1.0 mg/mL of each drug, dextromethorphan, levomethorphan, dextrorphan, 3-hydroxymorphinan, 3-methoxymorphinan, and levorphanol, was prepared in methanol and stored at 4 °C. The IS solutions of 1 μg/mL of levallorphan in methanol for the analysis of hair samples and those of 1 μg/mL of levallorphan in distilled water for plasma and urine samples were also prepared.

The drug concentrations in the samples were calculated using the peak–area ratios of the ions monitored for the target compounds versus IS. The calibration curves for the determination were constructed by analyzing extracted drug-free control samples spiked with the standard solution, as described above. The calibration samples containing 0, 1, 2, 4, 20, 40, 200, and 400 ng/mL of the target drugs for the rat plasma, 0, 5, 10, 50, 100, 500, 1,000 2,500, 5,000, and 10,000 ng/mL for the urine samples and 0, 0.1, 0.5, 1.0, 5.0, 10, 25, and 50 ng/mg for the hair samples were prepared just before analysis. The limit of quantitation (LOQ) of each drug was chosen to be the concentration of the lowest calibration standard with an acceptable limit of variance, while the limit of detection (LOD) was defined as concentrations in a sample matrix resulting in peak areas with signal-to-noise ratios (*S/N*) of 3.

The precision of the method was evaluated by five consecutive analyses of the plasma and urine samples that were spiked with the standard solutions containing 2, 20, and 200 ng/mL for the rat plasma samples and 5, 500, and 5,000 ng/mL for the urine samples, respectively. For the hair analyses, the control samples spiked with the standard solutions each containing 0.1, 5, and 50 ng/mg of the targeted drugs were evaluated. The recoveries of the four analytes from the rat samples were determined using each sample spiked with the analytes at a concentration of 80 ng/mL for the plasma, 500 ng/mL for the urine, and 10 ng/mg for the hair, respectively. To determine the recoveries, the responses of the analytes in the standard solutions and in the extracts from the rat control samples were compared. For the quantitative analysis of (−)-MEM and (−)-HM, the calibration curves of (+)-MEM and (+)-HM were used.

Demethylation of dextromethorphan/levomethorphan in rat and human liver microsomes

For the *in vitro* experiments with rat and human liver microsomes, the reaction mixture consisted of 0.1 M potassium phosphate buffer (pH 7.4) with an NADPH generating system (1.3 mM NADP, 3.3 mM G-6-P, 0.4 U/mL G-6-PDH, 3.3 mM $MgCl_2$), 50 μM substrate (dextromethorphan or levomethorphan), and 0.5 mg protein/mL microsomes (rat or human liver microsomes) in a final volume of 200 μL. Dextromethorphan and levomethorphan were dissolved in methanol, and the final concentration of the organic solvent was 0.1%. The incubation was started by adding the microsomal fraction and then continued for 0, 5, 10, or 20 min. The reaction was terminated by adding an equal volume of a mixed organic solution of 50% acetonitrile and 50% methanol, including 10 μM levallorphan (IS), and vigorous shaking. At the same time, a reaction mixture without the microsomal fraction was also incubated as an enzyme-free control. The mixture was centrifuged at 3,500×*g* for 3 min at 4 °C, and the supernatant was filtered prior to the injection for the LC-MS/MS analysis. The *in vitro* experiments for kinetic analyses were also performed as described above, except that 2, 5, 10, 50, 100, and 150 μM of substrates were incubated with the rat and human liver microsomes for 10 min. Each experiment was performed in duplicate and kinetic parameters were calculated with Eadie–Hofstee plots.

The results of the *in vitro* experiments were each evaluated by three consecutive analyses. The amounts of dextromethorphan/levomethorphan and their metabolites were calculated on the basis of calibration curves made by spiking known amounts of these compounds into the reaction mixture without the microsomal fraction.

Results

Chiral separation of dextromethorphan/levomethorphan and their metabolites

Complete chiral separation of dextromethorphan, levomethorphan, and their metabolites was achieved in 12 min on a Chiral CD-Ph column in 0.1% formic acid–acetonitrile by a linear gradient program. The retention time of each compound was as follows: the parent compounds (dextro/levo forms, 10.6/11.3 min) and their metabolites of *O*-demethyl (6.1/5.5 min), *N*-demethyl (8.1/9.8 min), and *O*, *N*-didemethyl (3.9/4.5 min) as shown in Table 1. Figure 3 shows LC-MS/MS total ion current chromatograms (MRM mode) of the extract from plasma (30 min after the first administration), urine (0–24 h after the last administration), and hair (collected 4 weeks after the first administration) of rats administered with dextromethorphan or levomethorphan. Under the chromatographic conditions used, there was no interference with any of the compounds or the internal standard by any extractable endogenous materials in the rat samples. The peaks 7 (9.8 min, *m/z* 258→170) and 8 (4.5 min, *m/z* 244→156) on the chromatograms shown in Fig. 3 were identified as those of (−)-3-MEM and (−)-3-HM when the mass fragmentations of these peaks were considered, although the standard compounds of these two metabolites were not available. These peaks were also confirmed by comparison of their retention times and mass fragmentations with those of the standard compounds of the dextro forms ((+)-3-MEM and (+)-3-HM) using an ODS column.

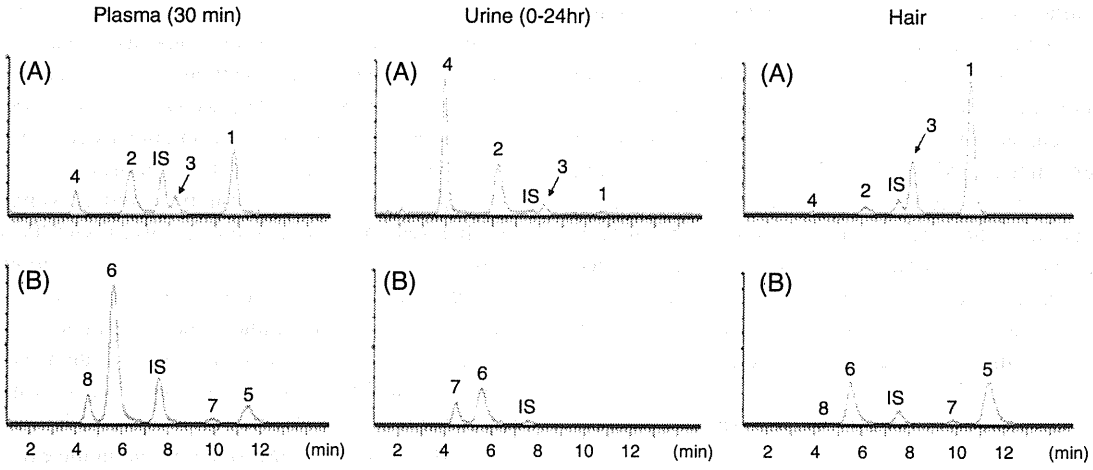


Fig. 3 LC-MS/MS total ion current (TIC) chromatograms (MRM mode) of the extracts from plasma, urine, and hair of rats administered with (**A**) dextromethorphan and (**B**) levomethorphan using a chiral column. *1* Dextromethorphan, *2* Dextrorphan, *3* (+)-3-MEM, *4* (+)-3-HM, *5* Levomethorphan, *6* Levorphanol, *7* (−)-3-MEM, *8* (−)-3-HM

Table 2 Validation of results of the LC-MS/MS analyses of dextromethorphan/levomethorphan and their metabolites in rat plasma, urine and hair samples (n=5)

Samples		Compounds	LOD (S/N>3)	LOQ (S/N>10)	Linear ranges	Recoveries (%)	Precision (%) (n=5)			Accuracy (%) (n=5)		
				(ng/mL)		80 ng/mL	2.0 ng/mL	20 ng/mL	200 ng/mL	2.0 ng/mL	20 ng/mL	200 ng/mL
Plasma (50 μL)	Dextro	Dextromethorphan	0.8	1.0	1.0-400	106.1	22.1	9.3	1.5	-19.2	5.5	-0.2
		Dextrorphan	0.4	0.8		81.7	10.2	3.8	1.5	10.2	2.2	-3.6
		(+)-3-MEM	0.8	1.0		110.5	15.0	3.2	2.5	23.5	2.1	2.6
		(+)-3-HM	0.8	1.0		92.5	15.7	6.1	1.8	13.7	-8.3	2.9
	Levo	Levomethorphan	0.8	1.0	1.0-400	100.8	8.6	4.9	2.5	21.6	-4.4	-5.7
		Levorphanol	0.8	1.0		90.7	15.9	4.1	2.3	-10.6	-5.6	-3.6
				(ng/mL)		500 ng/mL	5.0 ng/mL	500 ng/mL	5000 ng/mL	5.0 ng/mL	500 ng/mL	5000 ng/mL
Urine (100 μL)	Dextro	Dextromethorphan	1.0	2.5	5.0-10000	90.2	9.7	0.8	2.6	-4.8	-5.2	-4.8
		Dextrorphan	1.0	2.5		106.1	23.6	4.6	3.2	-17.9	11.1	-3.3
		(+)-3-MEM	2.5	5.0		102.5	19.7	6.1	4.2	10.4	-5.8	2.7
		(+)-3-HM	2.5	5.0		91.3	24.6	5.1	2.6	1.6	-9.9	1.5
							10 ng/mL			10 ng/mL		
	Levo	Levomethorphan	1.0	5.0	10-10000	94.6	10.9	9.5	2.6	-4.3	-17.0	-2.2
		Levorphanol	1.0	5.0		93.1	4.8	4.5	4.6	18.6	-8.0	6.8
				(ng/mg)		10 ng/mg	0.1 ng/mg	5.0 ng/mg	50 ng/mg	0.1 ng/mg	5.0 ng/mg	50 ng/mg
Hair (10 mg)	Dextro	Dextromethorphan	0.025	0.05	0.1-50	84.2	11.5	4.5	2.8	4.6	18.8	-6.6
		Dextrorphan	0.025	0.05		99.8	6.4	2.6	2.7	3.7	15.4	-3.5
		(+)-3-MEM	0.025	0.05		83.8	18.6	3.9	1.5	4.7	0.6	-2.2
		(+)-3-HM	0.025	0.1		91.4	11.2	6.2	2.8	4.6	18.8	-6.6
	Levo	Levomethorphan	0.025	0.1	0.1-50	98.1	9.9	9.8	5.5	0.1	-2.3	-5.1
		Levorphanol	0.025	0.05		112.2	8.8	2.8	4.2	11.9	1.3	-3.8

Linearity and precision of the analytical method for the rat urine, plasma, and hair samples

The calibration curves were linear over the concentration range 1.0–400 ng/mL for rat plasma, 5.0–10,000 ng/mL (compounds of dextro forms) and 10.0–10,000 ng/mL (compounds of levo forms) for rat urine, and 0.1–50 ng/mg for rat hair, with good correlation coefficients of $r^2 \geq 0.996$, respectively. The LOD of each drug was 0.4 or 0.8 ng/mL for the plasma, 1.0 or 2.5 ng/mL for the urine, and 25 pg/mg for the hair samples. The recoveries and the precision and accuracy data from the analytical procedures for the rat samples (n=5), spiked with a standard solution of the targeted compounds, are shown in Table 2.

Determination of dextromethorphan/levomethorphan and their metabolites in DA rat plasma, urine, and hair samples

It has been reported that a female DA rat lacks the CYP2D1 enzyme, which is known to be related to *O*-demethylation of dextromethorphan in the SD rat; it is therefore used as a model animal for the poor metabolizer phenotype of dextromethorphan [35–37]. As such, the metabolic data from female DA rats may not reflect the "normal" situation. On the other hand, pigmented hairy rats appear to be suitable for the investigation of analytical methods of basic drugs in hair samples, compared with albino rats (SD or Wistar rats) because pigmentation (the melanin contents) is one of the most important factors regarding the incorporation of basic drugs into hair, as described before [38]. Therefore, thus far, we have studied the analytical properties of various drugs in hair samples using the pigmented hairy male DA rats, avoiding female DA rats.

After the i.p. administration of dextromethorphan or levomethorphan to pigmented hairy male DA rats, the parent compounds and their three metabolites in the plasma, urine, and hair were determined using LC-MS/MS. The optical purities of the resulting metabolites were unchanged in any rat biological sample, and no racemation was observed through *O*- and/or *N*-demethylation (Fig. 3). In the rat plasma ($AUCs_{0-360\ min}$) and urine samples (total excretions for 0–72 h) after the hydrolysis of *O*-glucuronides, most metabolites were detected as being the corresponding *O*-demethyl and *N*, *O*-didemethyl compounds, as shown in Table 3. However, obvious differences in the amounts of these metabolites were found between the dextro and levo forms. After administration of dextromethorphan, dextrorphan and (+)-3-HM were the major metabolites in the plasma (59.4 and 64.3 mg/L·min) and urine (106.1 and 226.9 μg/mL). However, *O*-demethyl metabolites (levorphanol) were mainly detected in the plasma (197.1 mg/L·min) and urine (210.5 μg/mL) after administration of levomethorphan (Table 3).

In the hair samples, the differences in the amounts of the metabolites are more clearly detected. After administration of dextromethorphan, the parent compound and the *N*-demethyl metabolite ((+)-3-MEM) were mainly detected at 63.4 and 25.1 ng/mg, respectively, although the *O*-demethyl metabolite of dextromethorphan (dextrorphan) was detected at only 2.70 ng/mg, which was nearly one tenth of the level

Table 3 Rat plasma $AUCs_{0-360min}$, total excretion into rat urine, and concentrations in rat hair of dextromethorphan/levomethorphan and their metabolites

Administrations	Targeted compounds	Plasma $AUC_{0-360min}$ (mg/L·min)	Urine Total excretion 0–72 h (μg)	Hair Concentration (ng/mg)
Dextromethorphan (rat 1–3)	Dextromethorphan	23.8±17.6	2.13±1.05	63.4±4.6
	Dextrorphan	59.4±16.3	106.1±15.3	2.70±0.04
	(+)-3-MEM	3.10±2.15	6.95±0.68	25.1±1.9
	(+)-3-HM	64.3±31.3	226.9±51.3	0.70±0.11
Levomethorphan (rat 4–6)	Levomethorphan	6.90±5.12	0.59±0.61	24.5±5.3
	Levorphanol	197.1±48.2	210.5±36.2	24.6±2.4
	(−)-3-MEM	1.47±0.64	0.13±0.06	2.57±0.71
	(−)-3-HM	51.5±9.6	39.0±5.9	0.49±0.09

of levorphanol. In contrast, after the administration of levomethorphan, the parent compound and the *O*-demethyl metabolite (levorphanol) were mainly detected at 24.5 and 24.6 ng/mg, respectively, with a small amount of the *N*-demethyl metabolite ((−)-3-MEM). The *N*, *O*-didemethyl metabolites (3-HM) were hardly detected in either sample (Table 3). The ratios of the parent compounds, their *O*-demethyl, *N*-demethyl, and *N*, *O*-didemethyl metabolites in the hair samples were 100:4:40:1 for the dextro forms and 100:100:11:2 for the levo forms, respectively.

The rat plasma AUCs, total excretions into rat urine and concentrations in rat hair of dextromethorphan or levomethorphan, and their metabolites are summarized in Fig. 4. The metabolic ratios of dextromethorphan/levomethorphan, *O*-demethyl, *N*-demethyl, and *N*, *O*-didemethyl metabolites in rat plasma ($AUC_{0-360\ min}$) and hair (collected 4 weeks after the first administration) were 1:3:0.1:3 and 1:0.04:0.4:0.01 for the dextro forms and 1:29:0.2:7 and 1:1:0.1:0.02 for the levo forms, respectively. It is of interest that the concentrations of dextromethorphan and levome-

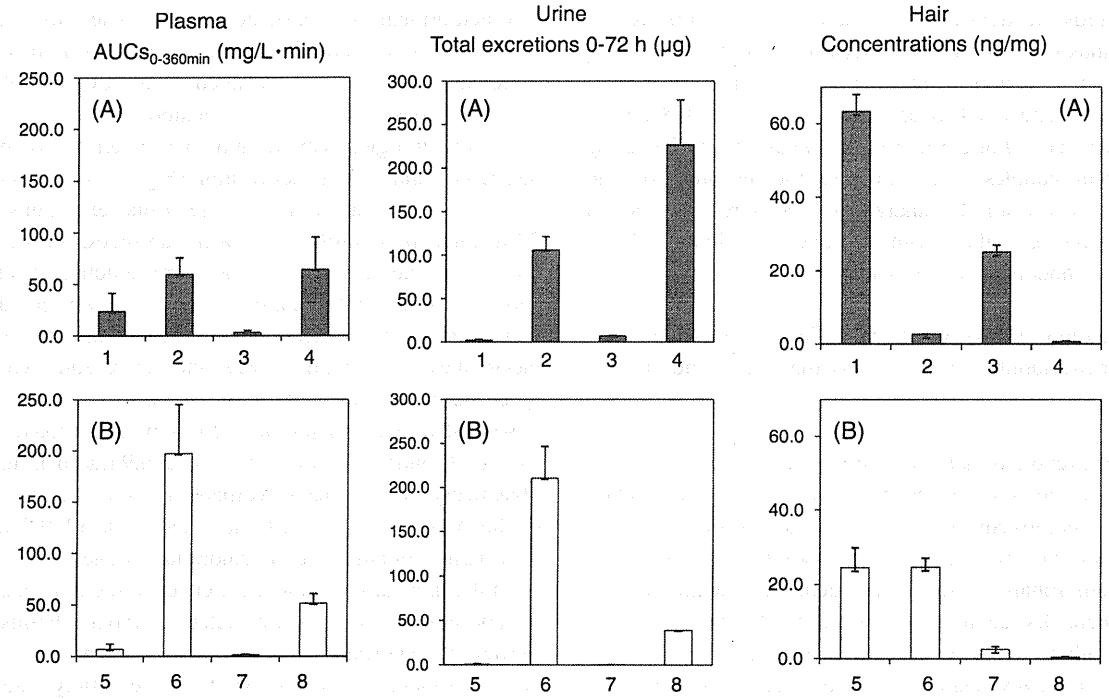


Fig. 4 Rat plasma $AUCs_{0-360min}$, total excretions into rat urine, and concentrations in rat hair of parent compounds and their metabolites after administration of (**A**) dextromethorphan and (**B**) levomethorphan. *1* Dextromethorphan, *2* Dextrorphan, *3* (+)-3-MEM, *4* (+)-3-HM, *5* Levomethorphan, *6* Levorphanol, *7* (−)-3-MEM, *8* (−)-3-HM

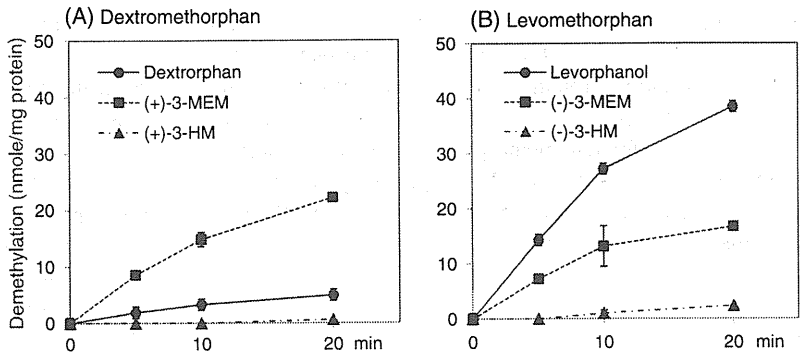


Fig. 5 Demethylation of (**A**) dextromethorphan and (**B**) levomethorphan in DA rat liver microsomes

thorphan in the rat hair were obviously high compared with those in the plasma, while those of their *O*-demethyl and *N*, *O*-didemethyl metabolites in the hair (which mostly existed as very hydrophilic metabolites, *O*-glucuronides in the plasma) were extremely low considering their high plasma AUCs.

Demethylation of dextromethorphan/levomethorphan in DA rat liver microsomes

In order to fully investigate the differences of the metabolic properties between dextromethorphan and levomethorphan, DA rat liver microsomes were studied. Figure 5 shows the *O*- and/or *N*-demethylation of dextromethorphan/levomethorphan in the rat liver microsomes.

The optical purities of the resulting metabolites were unchanged in the liver microsomes, and no racemation was observed through *O*- and/or *N*-demethylation. After 20-min incubation, 4.8% of dextromethorphan and 45% of levomethorphan were transformed to each *O*-demethyl metabolite, and 22% and 19% of the parent compounds were transformed to each *N*-demethyl metabolite. The *N*-demethylation was preferred over *O*-demethylation for dextromethorphan. In contrast, *O*-demethylation was preferred over *N*-demethylation for levomethorphan and the *O*-demethylation of levomethorphan was performed at levels 9.4 times that of dextromethorphan after 20-min incubation. The *N*-demethylation of levomethorphan was almost the same as that of dextromethorphan. Table 4 shows kinetic parameters for *O*-demethylation of dextromethorphan and levomethorphan by the DA rat microsomes. The V_{max} value for levomethorphan (3.8±0.3 nmol/min/mg protein) was 5.9 times higher than that of dextromethorphan (0.65±0.03 nmol/min/mg protein). The K_m values for levomethorphan and dextromethorphan were 22.1±5.0 and 44.1±4.0 μM, respectively. These results suggest that there might be an enantioselective *O*-demethylation of levomethorphan in the DA rat liver microsomes. This enantioselective metabolism might be the cause of the different amounts of the metabolites observed in the rat plasma, urine, and hair after administration of dextromethorphan and levomethorphan.

Demethylation of dextromethorphan/levomethorphan in pooled human liver microsomes

In order to investigate whether the enantioselective metabolism could be observed in humans as well as in DA rats, the pooled human liver microsomes were examined. Figure 6 shows the *O*- and/or *N*-demethylation of dextromethorphan/levomethorphan in the human liver microsomes.

The optical purities of the resulting metabolites were unchanged also in the human liver microsomes, and no racemation was observed through *O*- and/or *N*-demethylation. After 20-min incubation, 3.3% of dextromethorphan and 11% of levomethorphan were transformed to each *O*-demethyl metabolite and 2.5% and 7.1% of the parent compounds were transformed to each *N*-demethyl metabolite. The total amounts of the three metabolites from levomethorphan were higher than those from dextromethorphan in human (3.1 times) microsomes. Kinetic parameters for *O*-demethylation of dextromethorphan and

Table 4 Kinetic parameters for *O*-demethylation of dextromethorphan/levomethorphan by DA rat and human liver microsomes

	DA rat liver microsomes		Human liver microsomes	
	Dextromethorphan	Levomethorphan	Dextromethorphan	Levomethorphan
V_{max} (nmol/min/mg protein)	0.65±0.03	3.8±0.3[a]	0.26±0.03	0.58±0.02[a]
K_m (μM)	44.1±4.0	22.1±5.0[a]	4.5±0.9	8.9±1.7[a]

[a] Significantly different from dextromethorphan ($p<0.01$)

levomethorphan in the human liver microsomes are listed in Table 4. The V_{max} value for levomethorphan (0.58±0.02 nmol/min/mg protein) was 2.2 times higher than that of dextromethorphan (0.26±0.03 nmol/min/mg protein). The K_m values for levomethorphan and dextromethorphan were 8.9±1.7 and 4.5±0.8 μM, respectively. There could also be an enantioselective metabolism of levomethorphan in human liver microsomes.

Discussion

In this study, we first investigated the analytical methods of dextromethorphan/levomethorphan and their metabolites in biological samples using DA male rats. As a result, chiral separation of dextromethorphan, levomethorphan, and their metabolites in biological samples was achieved in 12 min on a Chiral CD-Ph column. The optical purities of the resulting metabolites were unchanged in all rat biological samples, and no racemation was observed through *O*- and/or *N*-demethylation. The proposed chiral analyses might be applied to human samples and could provide useful information for discriminating dextromethorphan use from levomethorphan use, considering the possibility of the adulteration or substitution of dextromethorphan with levomethorphan for illegal purposes. However, for application to forensic toxicological purposes, further studies should be carried out using authentic human samples.

The concentrations of dextromethorphan and levomethorphan in the rat hair were obviously high compared with those of metabolites in the plasma and urine samples in this study. In our previous study [38], we determined the melanin affinity and lipophilicity of 20 abused drugs and these values were compared with the ratio of drug concentration in hair to plasma AUC as an index of the incorporation tendency into hair. As a result, the combination of melanin affinity (basicity) and lipophilicity showed a high correlation with the incorporation tendency into hair. Parent compounds can be detected relatively easily in hair in comparison with their hydrophilic metabolites. Actually, it has been reported that cocaine is detected in hair at a much higher concentration than its metabolite, benzoylecgonine, although cocaine is rapidly hydrolyzed to benzoylecgonine and disappears from plasma [39]. Considering those reports, the physico-chemical properties of dextromethorphan/levomethorphan and their metabolites could be significantly related to their concentrations in the hair samples. Additionally, the drug concentrations in the rat hair (collected 4 weeks after the first administration) reflected the total amounts of drugs in the plasma of rats administered with dextromethorphan/levomethorphan for ten successive days, and the differences might become more distinct. The detection of the parent compounds from hair samples would provide useful information regarding the monitoring of their use over a long period.

In the DA rat samples, obvious differences in the ratios of the metabolites were found between the dextro and levo forms. These differences were most clearly detected in the hair samples. The concentrations of the parent compounds, their *O*-demethyl, *N*-demethyl, and *N*, *O*-didemethyl metabolites were 63.4, 2.7, 25.1, and 0.7 ng/mg for the dextro forms and 24.5, 24.6, 2.6, and 0.5 ng/mg for the levo forms, respectively. In order to investigate the differences of their metabolic properties between dextromethorphan and levomethorphan, DA rat and human liver microsomes were studied. As a result, we have shown the enantioselective metabolism of levomethorphan, not only in DA rats but also in human liver microsomes, especially with regards to the *O*-demethylation. Because it is well-known that CYP2D6 (mainly related to *O*-demethylation of dextromethorphan) is polymorphically expressed in humans, it may be difficult to discuss the enantioselective metabolism in humans who can be classified as poor, intermediate and extensive metabolizers of dextromethorphan. In future studies, the metabolic properties of these drugs using CYP2D6 enzymes (having a variety of phenotypes) should be examined to clarify the effects of their genotypes on the enantioselective *O*-demethylation of levomethorphan observed in this study.

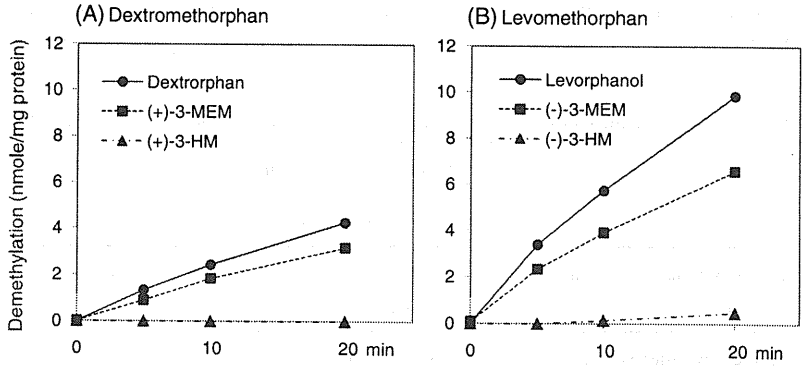


Fig. 6 Demethylation of (**A**) dextromethorphan and (**B**) levomethorphan in human liver microsomes

Conclusions

In this present study, we have established procedures for chiral analyses of dextromethorphan, levomethorphan, and their *O*-demethyl and/or *N*-demethyl metabolites in rat plasma, urine, and hair using LC-MS/MS. These analytical methods might be applied to human samples and could be useful for discriminating dextromethorphan use from levomethorphan use although further studies should be carried out using authentic human samples for forensic toxicological purposes. In addition, we have found the enantioselective metabolism of levomethorphan, not only in DA rats but also in human liver microsomes, especially with regards to the *O*-demethylation. This is the first report describing the differences in metabolic properties between dextromethorphan and levomethorphan in rats and humans.

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Effect of reducing the top concentration used in the *in vitro* chromosomal aberration test in CHL cells on the evaluation of industrial chemical genotoxicity

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ABSTRACT

A current concern with *in vitro* mammalian cell genotoxicity testing is the high frequency of false or misleading positive results caused in part by the past use of excessively high test concentrations. A dataset of 249 industrial chemicals used in Japan and tested for genotoxicity was analyzed. Of these, 116 (46.6%) were positive in the *in vitro* chromosomal aberration (CA) test, including 6 that were positive only at test concentrations >10 mM. There were 59 CA-positive chemicals at test concentrations ≤1 mM. At >1 mM, 51 chemicals were CA-positive, including 13 Ames-positive chemicals, which were therefore not "missed" by the test battery. Thus, 38 potentially positive chemicals would not have been detected in the test battery if the top test concentration was limited to 1 mM in CA test. Analysis of the relevance of CA results on the 38 missed chemicals was conducted based on a weight of evidence approach, including evaluations of effects of extreme culture conditions (low pH, high toxicity, or precipitation), *in silico* structural alert analysis, *in vivo* genotoxicity and carcinogenicity test data (where available), mode of action, or information from closely related chemicals. After an exhaustive review, there were four chemicals with some concern for human health risk assessment, nine with minimal concern, and the remaining 25 with negligible concern. We apply different top concentrations to the 38 missed chemicals to identify the most accurate approach for predicting the genotoxicity of industrial chemicals. Of these 2 mM or 1 mg/mL, whichever is higher, was the most effective in detecting these chemicals, *i.e.*, relatively higher (8/13) or lower (17/25) detection among 13 chemicals with some or minimal concern, or 25 with negligible concern, respectively. Lower top concentration limits, 1 mM or 0.5 mg/mL, whichever is higher, are not as effective (2/13) for detecting these chemicals with concern. Therefore, we conclude 2 mM or 1 mg/mL, whichever is higher, would be an appropriate top concentration limit for testing industrial chemicals for chromosome damage.

1. Introduction

The top concentrations used in *in vitro* mammalian cell genotoxicity tests are currently being re-examined with the goal of reducing the frequency of false or misleading positive results [1–6]. In the standard test battery, the mouse lymphoma assay and the *in vitro* micronucleus (MN) or chromosomal aberration (CA) test have low specificity for predicting carcinogenicity (e.g., <45%) [7–9], and the false positive results they generate lead to additional testing, often requiring the unnecessary use of animals [4]. The current top concentration limit specified in regulatory guidelines is 10 mM or 5 mg/mL, whichever is lower, when not limited by solubility or cytotoxicity [10,11]. The possible reasons for misleading or non-relevant results caused by testing at very high concentrations *in vitro* are: (1) un-physiological culture conditions including low pH, high osmolality and/or precipitation; (2) excessive cellular metabolic turn over, activation and defense/stress processes; and (3) results obtained at high concentrations that could not be reached *in vivo* and therefore not confirmed in *in vivo* genotoxicity or carcinogenicity tests. Therefore, more biologically relevant experimental conditions are needed. One way to lessen false positive results is to reduce the top test concentration [1]. The proposed revised International Conference on Harmonisation (ICH) test guidelines for pharmaceuticals recommends as the top concentration 1 mM or 0.5 mg/mL, whichever is lower [12], and almost same conclusion, *i.e.*,1 mM or 0.5 mg/mL, whichever is higher, was reached following an analysis of 384 genotoxic rodent carcinogens [3] and the retesting of selected chemicals [4]. A consensus for reducing the top concentration for testing in mammalian genotoxicity tests *in vitro* from 10 mM (but no agreement to what concentration should be) was reached in 2009 at the 5th International Workshop on Genotoxicity Testing, where the results from our preliminary analysis of 249 chemicals were presented [5]. Earlier in 2006, participants in a European Centre for the Validation of Alternative Methods workshop proposed that the published and industry data should

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be reviewed to determine whether the top test concentration should be lowered [1]. Therefore, our analysis will provide useful information for this debate.

As a member of the Organization for Economic Co-operation and Development (OECD) since 1991, Japan has been performing safety tests of high production volume (HPV) chemicals. The results of the 249 chemicals analyzed in this paper as part of that effort were published as hard copies [13–26], while recent results are available only online (http://dra4.nihs.go.jp/mhlw_data/jsp/SearchPageENG.jsp). The dataset includes results of the *in vitro* CA test and the Ames test, both conducted in accordance with OECD or Japanese test guideline and according to Good Laboratory Practice (GLP) regulations. In this paper, we report our analysis of "missed" chemicals—that is, those that were positive in the *in vitro* CA test at the 10 mM top concentration but not at 1 mM and were negative in the Ames test. We discuss the significance and relevance of the induction of CAs by these chemicals based on the weight of evidence approach including *in silico* analysis, review of literature on *in vivo* genotoxicity and carcinogenicity, or effects in structurally related chemicals. The levels of concern for human health risk assessment are defined for each chemical in this paper. Finally, we apply different top concentrations to missed chemicals to identify the most accurate approach for predicting the genotoxicity of industrial chemicals.

2. Materials and methods

2.1. Source of published in vitro CA test results

We obtained *in vitro* CA test data from reports published from 1994–2006 by the Ministry of Health, Labor, and Welfare of Japan [13–26].

2.2. In vitro CA test protocol

In vitro CA tests were conducted in Chinese hamster lung (CHL) cells in accordance with OECD test guideline no. 473 [11] or the Japanese test guideline for new chemicals [27] under GLP conditions. The outline of the protocol under the former Japanese test guideline were as follows: Treatment length was 6 h (with 18 h recovery), 24 h (continuous without recovery time), or 48 h (continuous without recovery time) without S9 mix, a rat liver homogenate microsomal fraction with co-factors for metabolic activation, and 6 h (with 18 h recovery) with S9 mix. The top concentration was 5 mg/mL (or equivalent of 10 mM) when no cytotoxicity was observed. In the presence of cytotoxicity, the top concentration selected was one that caused 50% or greater inhibition of cell growth compared to the negative control. Methods for measuring cytotoxicity, as relative cell growth, were an estimation of monolayer confluence using a monocellater or other equipment, or survival cell counts. Structural aberrations and polyploidy were evaluated independently in 100 or 200 metaphases per concentration. In some cases, 800 metaphases per concentration were analyzed for polyploidy. Pre-1997 studies did not follow the current OECD guideline that was published that year. Major differences from the current OECD guideline are use of long exposure time (48-h continuous treatment), use of concentrations which shows much greater than 50% relative cell growth as the top concentration when cytotoxicity was observed. More preferably 5 mg/mL was used, rather than 10 mM as the top concentration when cytotoxicity was not observed. In general, there was no consideration of physiological culture conditions (pH, osmolality, or precipitation), no concurrent cytotoxicity measurement, no ensuring that at least 200 metaphases were analyzed per concentration, and no statisticalanalyses were carried out [27,28].

2.3. Reporting of results

Each experiment was classified as (a) positive (+): ≥10% cells with CAs; (b) equivocal (?): ≥5–10% cells with CAs, or (c) negative (−): less than 5% cells with CAs. Then only chemicals showing at least one positive or equivocal experiment were considered positive or equivocal compound, respectively, in the original reports [13–26]. Statistical significance, reproducibility, culture conditions, or concentration relationship of the response were taken into consideration in some cases. Basically, calls of the classification of chemicals were based on the original calls. However, there are some exceptions to this analysis. If a chemical was classified equivocal in the original "call" and showed a reproducibility and/or concentration-related response with statistical significance, the chemical was considered positive. For example, two chemicals (Identifications (IDs) 61, 82) assigned equivocal in the original call were considered positive, because the effect was reproducible or there was a CA-induction of equal to or more than 10%. Three chemicals (IDs 81, 83, 84) assigned negative in the original call were also considered positive, because CA-induction of them was equal to or more than 10%; the original reports judged the CA-induction was due to low pH (see Section 3.1). Chemicals that show negative and/or equivocal (without reproducibility or concentration-related response) results were also considered as negative compounds.

Note that the percentages of cells with CAs refer to structural aberrations and do not include polyploidy. The percentages of polyploidy cells are presented in two chemicals (IDs 95, 96) in this analysis.

2.4. Analysis of the data

The different steps of the analysis (weight of evidence approach) used in this paper for the 249 chemicals on which *in vitro* CA tests were conducted, are shown in Fig. 1: (1) analysis of *in vitro* CA data excluding 48 h results; (2) classification of chemicals into positive and negative compounds; (3) for positive chemicals exclusion of those with the lowest effective concentration (LEC) of ≤1 mM or >10 mM; (4) for chemicals with LEC of >1–10 mM, review of Ames test data; if positive, chemicals would not be missed; (5) if Ames-negative (*i.e.*, possible "missed" chemicals in the test battery), further evaluation of the relevance of CA results, including evaluation of effects of extreme culture conditions (low pH, high toxicity and precipitation), *in silico* analyses using Deductive Estimation of Risk from Existing Knowledge (DEREK) for Windows and/or the Optimized Approach Based on Structural Indices Set (OASIS) tissue metabolic simulator (TIMES), and review of the literature to see if more *in vivo* genotoxicity and carcinogenicity data including on structurally related compounds can be found; and (6) conclusion on level of concern for human health risk assessment on missed chemicals.

2.5. In silico structure alert analysis

We used DEREK for Windows (version 12) for structure alerts for mutagenicity, clastogenicity, and carcinogenicity [29] and TIMES (version 2.26.3) for structural alerts for clastogenicity [30]. TIMES can predict CAs induced by metabolically activated chemicals that do not elicit activity in the parent form, in addition to alerting for mutagenic structures [30–33]. Basically, DEREK was applied to all "missed" chemicals. TIMES was also applied missed chemicals with the exception of chemicals which were considered positive due to possible effects of extreme culture conditions.

2.6. Literature search

For the literature search, we used *PubMed* and *TOXNET* and searched for "CAS number", "carcinogenicity", "genotoxicity", "mutagenicity", "micronucleus" and "chromosomal aberration". Data from structurally related chemicals were also taken into account. We also searched Screening Information Data Set (SIDS) documents in the United Nations Environment Programme (UNEP, http://www.chem.unep.ch/irptc/sids/OECDSIDS/sidspub.html) or the OECD chemical database (http://webnet.oecd.org/hpv/ui/Search.aspx) [34–53].

2.7. Level of concern

The level of concern for human health risk assessment on "missed" chemicals was defined based on the analysis by weight of evidence approach. General criteria are as follows: (1) negligible concern, negative result(s) in *in vivo* genotoxicity or carcinogenicity test, clear evidence(s) of irrelevancy (e.g., extreme culture condition) for CA-induction, and/or mode of action of non-DNA target; (2) minimal concern, some evidence(s) of irrelevancy of CA-induction or of increasing level of negligible concern, or negative result(s) in *in vivo* genotoxicity tests with some limitations; (3) some concern, positive result(s) in *in vivo* genotoxicity or carcinogenicity test, or no supporting evidence(s) for reducing the level of concern.

2.8. Application of different top concentrations to the "missed" chemicals

Several top concentration limits were applied to the "missed" chemicals in order to investigate their effectiveness for predicting the genotoxicity of industrial chemicals, which include 1 mM or 0.5 mg/mL, whichever is higher; 2 mM or 1 mg/mL, whichever is higher; 4 mM or 2 mg/mL, whichever is lower; and 10 mM or 2 mg/mL, whichever is lower. These top concentrations except for 2 mM or 1 mg/mL, whichever is higher, are under discussion by an OECD expert group (unpublished). The number of chemicals detected among missed chemicals with minimal or some concern, or with negligible concern was calculated.

3. Results

3.1. Analysis of in vitro CA and Ames test data

At first, we regarded two chemicals, benzyltrimethylammonium chloride (ID61, Table 2) and glycerol triacetate (ID82, Table 4), as positive in this analysis; their original "call" were equivocal [16,18], but the effects observed by benzyltrimethylammonium chloride or glycerol triacetate were reproducible or induced more than 10%