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ECVAM retrospective validation of *in vitro* micronucleus test (MNT)

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In the past decade several studies comparing the *in vitro* chromosome aberration test (CAT) and the *in vitro* micronucleus test (MNT) were performed. A high correlation was observed in each of the studies (>85%); however, no formal validation for the micronucleus *in vitro* assay had been carried out. Therefore, a working group was established by the European Centre for the Validation of Alternative Methods (ECVAM) to perform a retrospective validation of the existing data, in order to evaluate the validity of the *in vitro* MNT on the basis of the modular validation approach. The primary focus of this retrospective validation was on the evaluation of the potential of the *in vitro* MNT as alternative to the standard *in vitro* CAT. The working group evaluated, in a first step, the available published data and came to the conclusion that two studies [German ring trial, von der Hude, W., Kalweit, S., Engelhardt, G. *et al.* (2000) In-vitro micronucleus assay with Chinese hamster V79 cells: results of a collaborative study with 26 chemicals. *Mutat. Res.*, 468, 137–163, and SFTG International Collaborative Study, Lorge, E., Thybaud, V., Aardema, M., Oliver, J., Wataka, A., Lorenzon, G. and Marzin, D. (2006) SFTG International Collaborative Study on in-vitro micronucleus test I. General conditions and overall conclusions of the study. *Mutat. Res.*, 607, 13–36] met the criteria for a retrospective validation according to the criteria previously defined by the working group. These two studies were evaluated in depth (including the reanalysis of raw data) and provided the information required for assessing the reliability (reproducibility) of the test. For the assessment of the concordance between the *in vitro* MNT and the *in vitro* CAT, additional published data were considered. Based on this retrospective validation, the ECVAM Validation Management Team concluded that the *in vitro* MNT is reliable and relevant and can therefore be used as an alternative method to the *in vitro* CAT. Following peer review, these conclusions were formally endorsed by the ECVAM Scientific Advisory Committee.

Introduction

The *in vitro* micronucleus test (MNT) is used by academics, industry and contract laboratory organizations for internal hazard identification and compound prioritization as an alternative/replacement of the *in vitro* chromosome aberration test (CAT). This test has already gained widespread international interest, as it offers significant advantages over the *in vitro* CAT. The *in vitro* MNT allows the detection of both clastogens and aneugens and it can simultaneously detect mitotic delay, apoptosis, chromosome breakage, chromosome loss and non-disjunction (1,2). Different end points can be regarded and used as biomarkers of DNA damage and therefore the assay can be referred to as micronucleus cytome assay providing additional important mechanistic information (3). Furthermore, the scoring phase can be automated by flow cytometry and/or image analysis which benefits screening programmes. The *in vitro* MNT is a simple test method. Slide reading is easy, more objective and quick, resulting in a much higher throughput. Metaphase analysis is in contrast very tedious, time consuming and has a low throughput. The *in vitro* MNT has been shown to be a robust test which can be applied to any type of primary cells or cell lines (4). Finally, it has greater accuracy and statistical power as thousands of cells can be scored compared to a few hundred in the *in vitro* CAT. A limitation of the *in vitro* MNT is that the assay does not provide information about the types of structural chromosome aberrations.

Although an extensive amount of published data is available to support the validity of the *in vitro* MNT using various cell lines, primary cells or human lymphocytes, the *in vitro* MNT assay is not yet generally accepted by regulatory authorities as an alternative system in a test battery. One of the reasons is that the *in vitro* MNT had not been formally validated. Therefore, following a recommendation of an expert meeting on the *in vitro* MNT held at the European Centre for the Validation of Alternative Methods (ECVAM) in 2004, ECVAM conducted this retrospective validation of the *in vitro* MNT. At the meeting, the experts agreed that, given the considerable data on the test already available and the high interest in using the test for regulatory purposes, an evaluation of the existing data should be undertaken to assess the validity on the *in vitro* MNT as alternative to the *in vitro* CAT.

In order to evaluate whether the test met all data requirements requested by the ECVAM principles on test validity, the modular approach of validation was followed (5). This approach is defined by seven validity modules: (1) test definition, (2) within-laboratory reproducibility, (3) transferability, (4) between-laboratory reproducibility, (5) predictive capacity, (6) applicability domain and (7) minimum performance

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standards. While module 1 describes the test, modules 2–4 cover the reproducibility aspects of the assay, module 5 the predictivity/concordance, module 6 the applicability domain and module 7 defines the requirements to accept additional data/assays for the same end point. Module 7 was not considered, as this is a retrospective evaluation of data.

Here, we present the evaluation undertaken by the Validation Management Team (VMT) established by ECVAM, which led to the conclusions that the *in vitro* MNT is a reproducible and reliable method to be used as an alternative to the *in vitro* CAT. An official validation report had been submitted earlier to ECVAM's Scientific Advisory Committee (ESAC), composed of representatives from all European Union Member States, academia, industry and animal welfare organizations for a peer review of the scientific validity of the *in vitro* MNT [for ESAC statement see (6)].

Material and methods

Several studies (4,7–16) were discussed and evaluated by an Expert Group during a meeting held at ECVAM, Italy, in April 2004. The analysis was mainly based on the criteria for protocol requirements defined by the Expert Group (Supplementary Material, Appendix 1 is available at *Mutagenesis* Online), the International Workshop on Genotoxicity Tests (IWGT) testing recommendations (4) and the Organization for the Economical Cooperation and Development (OECD) draft guideline [Test Guideline (TG) 487].

In the end, two data sets, the German ring trial (7) and the SFTG Ring Trial (8,13–16) were considered to meet most, but not all, of the set criteria for the ECVAM retrospective validation. Both studies were suitable for the analysis of the within-laboratory reproducibility, the transferability and the between-laboratory reproducibility, mainly due to their well controlled study set-up and the availability of the raw data for an in-depth expert re-evaluation. The main characteristics of the two studies are summarized in the Supplementary Material, Appendix 2 (available at *Mutagenesis* Online). All other studies cited above were only considered in the assessment of the concordance between the *in vitro* MNT and the *in vitro* CAT and were used to support/strengthen or negate the conclusions drawn by the VMT.

Evaluation of the studies for the assessment of reproducibility

The two studies used for re-evaluation of raw data were originally designed to address different purposes. The aim of the German ring trial was mainly to analyse the concordance between the *in vitro* MNT and the *in vitro* CAT. As it was designed similarly to a prospective validation, a standardized protocol was used in all participating laboratories. The main focus of the SFTG trial was to assess the optimal protocol design and the reproducibility of different protocols in several cell types. The raw data of the two studies were evaluated originally by different expert groups. As a consequence, the criteria considered for a positive call were not the same. In the German trial, biological relevance, a concentration-related increase of the micronuclei (MN) frequency and reproducibility of effects were the primary criteria for a positive call. In the SFTG study, the primary criteria were a concentration-related increase of MN frequency and a statistically significant increase in the incidence of micronucleated cells in treated samples over the solvent control. Taking the above factors into account, it was evident that the data set was heterogeneous in a way, which would complicate the comparison of data between studies. For this reason and in order to acquire more confidence in the data, it was considered necessary to reanalyse the raw data of both studies. The use of identical evaluation criteria led to a consistent call for both sets of raw data, allowing an improved final evaluation of the results.

The reanalysis of the raw data was conducted at ECVAM by experts who had not been involved in the two studies. A consensus on the criteria for a positive, negative and equivocal call was reached among the experts prior to the evaluation of the raw data. The criteria were determined by taking into account the following: (i) the criteria initially defined by the expert group as if they had to be applied in a prospective study, in a best case scenario (Supplementary Material, Appendix 1 is available at *Mutagenesis* Online); (ii) the criteria defined in the draft OECD TG on the *in vitro* MNT (TG 487) and (iii) the raw data available.

An early draft of the OECD TG 487 recommended the use of a test concentration that produces up to 60% cytotoxicity. However, in the past, concentrations which induce 50% cytotoxicity were used. Therefore, the VMT decided to evaluate the raw data considering both 50 and 60% cytotoxicity, allowing comparison of these two cytotoxicity criteria (Supplementary Material, Appendix 4 is available at *Mutagenesis* Online). For the purpose of the validity assessment of the *in vitro* MNT, only 60% cytotoxicity was considered. Measures considered in the assessment of cytotoxicity were proliferation index, mitotic index, viable cell count and, in the presence of cytochalasin B, percentage of multinucleated cells.

Criteria for the evaluation of raw data and the judgement of the relevance of effects

At the first meeting, the expert group agreed on a series of evaluation criteria as if they had been defined for a prospective validation exercise. However, for this retrospective validation exercise, not all criteria could be applied in every case. Consequently, the criteria were overruled by an independent expert judgement of the raw data.

Judgement of the biological relevance of the effects observed was applied primarily as the criterion to evaluate the data. This is in line with the main criteria to be considered according to the OECD TG for *in vitro* CAT and the draft guideline for *in vitro* MNT. The measure to assess the biological relevance of effects was the occurrence of a dose relationship and the magnitude of the effects. Statistical methods may be used as an aid in evaluating the test results (OECD guidelines for *in vitro* systems). Statistical significance was not considered in this re-evaluation of raw data as it was available only for the SFTG trial.

Historical control data were not available for the studies, which made it difficult to judge the relevance of the relative increases of MN compared to controls. However, the observed range of the negative controls (NCs) for each laboratory in this series of experiments was used as an aid when judging the relevance of effects.

A compound was called 'positive' if it clearly showed a dose-related increase in MN frequency and the upper limit of the observed range of NC for each laboratory had been exceeded. Likewise, a compound was 'negative' in the *in vitro* MNT when there was no dose-related increase in MN frequency and the upper limit of the observed range of NC for each laboratory had not been exceeded. If the use of the above-described criteria did not allow judging the individual experiment in question as positive, but the magnitude of the effect or the observed dose relationship questioned the classification of the test item as negative, the study was rated 'equivocal'. If in a study the required level of toxicity (50 or 60%) was not reached and no positive response was obtained, the study was rated as 'not appropriate' because it could not be excluded that at a higher level of toxicity a positive result would have been obtained.

To be in line with both the draft OECD TG and the current protocol requirements, the evaluation criteria in this evaluation were stricter than those used in the respective papers. Therefore, following the re-evaluation of the raw data, many experiments were re-categorized as not appropriate because at the time it was not required to test up to the currently requested levels of cytotoxicity (at least 50%). A summary of the number of experiments, which were not appropriate according to the defined criteria, is shown in Supplementary Material, Appendix 2 (Table A5, available at *Mutagenesis* Online).

In the SFTG study, the judgement was based on binucleated cells, if results in both binucleated and mononucleated cells were available. This allowed the comparison of results between all cell lines, including human lymphocytes. As in the German trial, data on both proliferation index and mitotic index were not consistently available, both parameters were considered equally adequate for the determination of cytotoxicity.

An overview of the treatments and recovery times used in the two studies is shown in Supplementary Material, Appendix 2 (Table A4, available at *Mutagenesis* Online). For the schematic representation of all data collected and re-evaluated by the VMT, see Supplementary Material, Appendix 2 (Table A6, available at *Mutagenesis* Online).

Evaluation of the available data for the assessment of concordance between the in vitro MNT and the in vitro CAT

The purpose of this retrospective validation is to determine whether the MNT *in vitro* can be used as alternative to the *in vitro* CAT. The assessment of concordance was based on the following studies and reviews of published data selected by the expert group and the VMT (7,9–12).

MNT data. The *in vitro* MNT data of the German trial, reported in Table III, represent the conclusions of the re-evaluation by the VMT. Regarding the study of Miller *et al.* (10), the data reported represent the conclusions of the Gesellschaft für Umwelt-Mutationsforshung (GUM) working group. For the other studies, the data are reported as they were published in the original papers. The data retrieved from the CGX database [(12), http://www.lhasalimited.org/cgx] were filtered out for the studies already described in the other data sources considered, in order to avoid duplications.

CAT data. The data for *in vitro* CAT were reviewed by D. Kirkland (Covance, UK), based on the published literature and expert judgement. In order to allow

a comparison of the two tests, the review was based on criteria that were as close as possible to those currently required for evaluation of *in vitro* CAT under regulatory testing and therefore comparable to the criteria used for the evaluation of the *in vitro* MNT in the studies considered. To achieve this, the results of old studies were evaluated according to the current testing requirements for genotoxicity testing (e.g. no test carried out above 10 mM). In addition, if *in vitro* CAT was concluded negative, but only performed in the absence of S9, these studies were considered inadequate for such a conclusion and were designated technically compromised. Such results could not be compared with the *in vitro* MNT results. In order for these judgements to be made, the original papers (or the NTP database in the case of NTP studies) were reviewed. Where necessary, literature searches were made through ToxLine and PubMed in order to uncover other publications. Information on numerical chromosome aberrations was, if available, included in Table III.

In cases with more than one experiment per compound, a positive result (both in the presence and in the absence of S9) always overruled a negative, equivocal or inconclusive result. Only when several negative results where obtained together with only one positive result, the final conclusion was inconclusive. In case of negative results together with an equivocal or inconclusive result, the final conclusion was 'inconclusive'.

Classification of the compounds. All compounds reported in Table III were classified into the following classes: clastogens, aneugens and non-genotoxic substances. When the information was available, the compounds were also classified as non-carcinogens. The classification was based on the available information present in the public domain and on expert judgement. The original papers (or the NTP database in the case of NTP studies) were reviewed and, where necessary, literature searches were made through ToxLine and PubMed in order to uncover other publications that would define the predominant types of activity. For some chemicals, the classification of aneugen could only be drawn from studies on non-mammalian systems such as on yeasts or other fungi. Some chemicals that were quite weak clastogens (inconsistent responses reported in literature or producing only borderline responses) were found to be clearly more genotoxic in other tests for mutational end points, such as the Ames or mouse lymphoma tests, and these are marked as such in Table III. The classification of the compounds was essentially carried out by D. Kirkland and was subsequently reviewed by the genotoxicity Roche expert group. The classification allowed evaluating the *in vitro* MNT–CAT concordance overall, as well as for each class of compounds separately.

Results

Within-laboratory reproducibility

The within-laboratory reproducibility assessment was based on the expert re-evaluation of raw data (Supplementary material, Appendix 2, Table A6 is available at *Mutagenesis* Online), which took into account the 60% cytotoxicity criterion.

Repeat experiments were conducted in most of the laboratories involved in the SFTG study and in some laboratories involved in the German study (in certain instances up to four times), allowing for the assessment of within-laboratory reproducibility. It was considered appropriate to conduct a descriptive analysis (based on biological relevance) of the data instead of a statistical one. One reason being the limited number of data points per each parameter.

Table I shows the within-laboratory reproducibility calculated for each treatment protocol and each cell line used in identical and independent experiments conducted in the same laboratory. For this evaluation, not appropriate data and equivocal data were excluded as it was assumed that in a prospective study (or in real life), experiments with results being not appropriate or equivocal would have been repeated.

When the evaluation was carried out for each cell model and treatment protocol, the within-laboratory reproducibility ranged from 83 to 100%. The lowest value was found for the L5178Y cells with the 'Long Long' treatment/recovery. The within-laboratory reproducibility assessed per treatment, independent from cell model, varied from 94 to 100%, while the reproducibility per cell line, independent from treatment, varied from 97 to 100%.

Between-laboratory reproducibility

The between-laboratory reproducibility was based on the expert conclusion of the raw data re-evaluation, as in the case of the within-laboratory reproducibility, and was assessed taking into account the 60% cytotoxicity criterion.

Since most of the laboratories repeated the identical experiment more than once, the following criteria were defined to reach a final conclusion when the results of an identical experiment conducted in the same laboratory were not concordant: (i) in the case of a positive and an equivocal experiment in the same laboratory, the final conclusion was positive and (ii) in the cases of negative and equivocal results or positive and negative results, the final conclusion was inconclusive (Supplementary material, Appendix 2, Table A7 is available at *Mutagenesis* Online).

It has to be noted that in a retrospective validation, which is based on published data, it is difficult to achieve a balance between clastogenic and non-clastogenic compounds. This literature bias is due to the publication of predominantly positive results. However, from the industry experience, it is known that the negatives are correctly predicted (9).

The data on the between-laboratory reproducibility per treatment protocol and per cell system are reported in Table II. Not appropriate, inconclusive and equivocal data were excluded, since in a prospective study (or in real life), an experiment with such results would have to be repeated. The

Table I. Within-laboratory reproducibility for each treatment and each cell system (exclusion of not appropriate and equivocal data)

	SFTG ring trial							German ring trial		
	Without CB				With CB			Without CB		
Treatment	S	S	L	L	S	S	L	L	S + S9	
Recovery	S	L	N	L	S	L	L	N	S	
HL	–	–	–	–	8:8, 100%	7:7 100%	6:6, 100%	–	–	21:21 100%
L5178Y	4:4, 100%	6:6, 100%	5:5, 100%	6:6, 100%	4:4, 100%	–	5:6, 83%	–	–	30:31 97%
CHL	13:13, 100%	8:8, 100%	11:12, 92%	6:7, 86%	9:9, 100%	–	10:10, 100%	–	–	57:59, 97%
CHO	7:7, 100%	5:5, 100%	4:4, 100%	5:5, 100%	6:6, 100%	–	5:5, 100%	–	–	27:27, 100%
V79	–	–	–	–	–	–	–	12:12, 100%	–	12:12, 100%
	24:24, 100%	19:19, 100%	21:21, 95%	17:18, 94%	27:27, 100%	7:7, 100%	26:27, 96%	12:12, 100%		

S, short; L, long; N, no recovery; CB, cytochalasin B. Reference to Table A4 (available at *Mutagenesis* Online) for details on the treatment and recovery times: HL, human lymphocytes; L5178Y, mouse lymphoma cells; CHL, Chinese hamster lung cells; CHO, Chinese hamster ovarian cells and V79, Chinese hamster lung fibroblasts.

Table II. Between-laboratory reproducibility for each treatment and each cell system (exclusion of not appropriate, equivocal and inconclusive data)

	SFTG ring trial							German ring trial		
	Without CB				With CB			Without CB		
Treatment	S	S	L	L	S	S	L	L	S + S9	
Recovery	S	L	N	L	S	L	L	N	S	
HL	–	–	–	–	3:3, 100%	5:5, 100%	5:5, 100%	–	–	13:13, 100%
L5178Y	1:1, 100%	3:3, 100%	2:3, 67%	2:3, 67%	1:2, 50%	–	2:2, 100%	–	–	11:14, 79%
CHL	5:5, 100%	4:4, 100%	5:5, 100%	2:2, 100%	4:4, 100%	–	5:5, 100%	–	–	25:25, 100%
CHO	5:5, 100%	5:5, 100%	4:4, 100%	3:3, 100%	3:3, 100%	–	4:4, 100%	–	–	24:24, 100%
V79	–	–	–	–	–	–	–	16:18, 89%	2:2, 100%	18:20, 90%
	11:11, 100%	12:12, 100%	11:12, 92%	7:8, 86%	11:12, 92%	5:5, 100%	16:16, 100%	16:18, 89%		

S, short; L, long; N, no recovery; CB, cytochalasin B; HL, human lymphocytes; L5178Y, mouse lymphoma cells; CHL, Chinese hamster lung cells; CHO, Chinese hamster ovarian cells and V79, Chinese hamster lung fibroblasts.

table presents the number and the percentage of laboratories which gave reproducible results for each treatment and each cell system, indicating also the number of chemicals eligible for this analysis. The data reported refer to the experiments that have been conducted in at least two laboratories. Only the laboratories that conducted identical experiments at least two times were considered.

The between-laboratory reproducibility assessed per treatment, independent from cell line, varied between 86% (for Long Long treatment) and 100%. The between-laboratory reproducibility assessed per cell model, independent from treatment, varied from 79% (for L5178Y) to 100%. Overall, taking into account all cell models and the different treatments, the between-laboratory reproducibility was 95%. Comparable reproducibility results were observed when different treatment protocols or cell lines were used, underlining the robustness of the assay.

Transferability

In general, the test method can easily be performed in a laboratory that is experienced in routine cell culture techniques. No particular facilities are required. General cell culture laboratory equipment and instruments are sufficient to perform the *in vitro* MNT. All supplies and reagents are readily available on the market. As stressed in the *in vitro* MN testing requirements, when human lymphocytes, are used they should derive from non-smoking, young, healthy donors. The *in vitro* MNT requires personnel trained for general cell biology and cell culture activities (e.g. aseptic operations). The operator should, in particular, be trained in the scoring of micronuclei. However, the training requirements for a person to be competent in scoring the slides are much less rigorous for *in vitro* MNT than for metaphase analysis. As there is no requirement to count the chromosomes in a metaphase preparation or to evaluate subtle chromatid and chromosome damage, but only to determine whether or not a cell contains a micronucleus, the scoring is faster and the evaluation is more objective.

The successful transferability of the MNT *in vitro* is demonstrated by the satisfactory results for the between-laboratory reproducibility from the studies evaluated, which included several naive laboratories.

Concordance analysis

In order to evaluate the overall concordance between the *in vitro* MNT and the *in vitro* CAT, all data on the substances tested both with *in vitro* MNT and *in vitro* CAT in the considered studies were summarized in a single table (Table III). The table also reports the type of cells used for the test and whether the test was performed in the presence or absence of S9.

The studies considered in this analysis differ in several characteristics such as the availability of raw data, whether or not the *in vitro* MNT and *in vitro* CAT were conducted in parallel within the same study, the quality of *in vitro* CAT reference data considered, the use of proprietary compounds or the number of compounds tested. As mentioned above, the concordance between *in vitro* MNT and *in vitro* CAT was analysed in each study separately and in addition by pooling all data (Table VIII). Important information about the different data sets considered and the concordance results for each of the studies are described in Supplementary material, Appendix 3 (available at *Mutagenesis* Online).

In Table IV, the concordance analysis for the 113 compounds of Table III is shown. The concordance between both assays was 83.2%. However, of the 92 *in vitro* MNT-positive compounds, 9 were negative in the *in vitro* CAT assay. Of these *in vitro* CAT-negative compounds, six are known as pure aneugens. Consequently, they were correctly negative in the *in vitro* CAT assay. Correcting the concordance for these aneugens, the capacity of the *in vitro* MNT to predict clastogens and aneugens was 88.5%.

Moreover, to allow a concordance analysis for each chemical class, all compounds were classified in the following classes: clastogens, aneugens and non-genotoxic (Tables V–VII). While the concordance between *in vitro* MNT and *in vitro* CAT is 77.8% for aneugens, the predictive capacity of MNT was 100% for the set of aneugenic compounds evaluated. The concordance for clastogens and non-genotoxins was 87.3 and 73.3%, respectively.

Table VIII summarizes the analysis of the performance of the *in vitro* MNT in comparison to *in vitro* CAT overall for the different classes of compounds and for each study. The concordance for the different studies ranged between 80.8 and 88.9%.

Discussion

The primary focus of this ECVAM retrospective validation using the modular validation approach (5) was to evaluate the potential of the *in vitro* MNT to serve as alternative to the standard *in vitro* CAT. Based on the data presented and evaluated, the ECVAM VMT concluded that the *in vitro* MNT meets all data requirements requested by the ECVAM

Table III. *In vitro* MNT and *in vitro* CAT results for all chemicals considered in the validation study (including class of chemicals)

Class	Chemical	CAS No.	S9 mix	Cells, MNT	MNT	Overall MNT	S9	Cells, CAT	CAT		Overall CAT	References, MNT	References, CAT
									SA	NA			
CL	Acetaminophen	103-90-2	−	CHL	+	+	−	Several	+	−	+	(11)	(17)
CL	Acetylsalicyclic acid (aspirin)	50-78-2	−	CHL	Weak +	+	−	CHL	+	−	+	(11)	(17)
CL	2-Acetylaminofluorene	53-96-3	+	L5178Y	+	+	−/+	CHL	+		+	(18)	(17)
			−	Several	+		−	RL1	+			(10)	(17)
			+	CHL	+		−	V79	−			(10)	(17)
			+	V79	+							(7)	
CL	Actinomycin D	50-76-0	−/+	CHO	+	+	−	Several	+		+	(19)	(17)
			−	Several	+		−	Several	+			(10)	(10)
CL	Adriamycin	25316-40-9	−	Several	+	+	−	Several	+		+	(10)	(10)
CL	Aflatoxin B1	1162-65-8	−	MCL-5	+	+	+	V79	+		+	(20)	(17)
			−	Several	+		−	Several	+			(10)	(10)
			+	HULY	+		+	HULY	+			(10)	(10)
CL	2-Aminoanthracene	613-13-8	−	Several	i	+	+	CHO	E		E	(10)	NTP database
			+	Several	+							(10)	
CL	2-Amino-3,4-dimethylimidazo -[4, 5-*f*]quinoline	77094-11-2	+	CHL	+	+	Not given	CHL	+		+	(11)	(11)
CL	2-Amino-1-methyl-6-phenylimidazo-[4, 5-*b*]pyridine	105650-23-4	+	CHL	+	+	Not given	CHL	+		+	(11)	(11)
CL	2-Amino-3-methyl-9*H*-pyrido-[2, 3-*b*] indole acetate	63170-60-5	−	CHL	+	+		Not available	+	+	+	(11)	(21)
CL	*m*-Amsacrine	54301-15-4	−	Several	+	+	−	Several	+		+	(10)	(10)
CL	Aniline	62-53-3	+	CHL	+	+	+	CHL	+	−	+	(11)	(17)
CL	*o*-Anthranilic acid	118-92-3	+	CHO	+	+	+	CHO	E		E	(22)	NTP database
NG/NC	L-ascorbic acid	50-81-7	−	CHO	+	+	−/+	CHO	−		−	(23)	NTP database
CL	Barbital	57-44-3	−	CHL	Weak +	+	−	CHL	+	−	i	(11)	(17)
							−	DON	−				(17)
CL	Benzene	71-43-2	+	CHL	Weak +	i	−/+	Several	+	−	+	(11)	(17)
			+	V79	−							(7)	
CL	Benzidine	92-87-5	−	MCL-5	+	+	−/+	Several	+		+	(20)	(17)
CL	Benzoin	579-44-2	+	CHL	Weak +	+	+	CHL	+	−	+	(11)	Concurrent test[a]
CL	Benzo[*a*]pyrene	50-32-8	+	CHL	+	+	+	CHL	+	−	+	(11)	Concurrent test[a]
			−	SHE/3T3	+		−	Several	−			(10)	(10)
			+	Several	+		+	Several	+			(10)	(10)
CL	Benzylchloride	100-44-7	−	CHL	+	+	−	CHO	+		+	(11)	(17)
							−	RL4	+				(17)
							−	HULY	−				(17)
NG/C	Benzylacetate	140-11-4	−	V79	i	i	−/+	CHL	−		−	(7)	(24)
CL	Bleomycin sulphate	11056-06-7	−	V79	+	+	−	Several	+		+	(7)	(17)
			−	Several	+				+			(10)	(10)
NG/C[b]	*N*-butyl-*N*-(3-carboxypropyl)nitrosamine	38252-74-3	−	CHL	Weak +	+	−	CHL	−	−	−	(11)	(17)
CL	Cadmium acetate	543-90-8	−	CHL	+	+	−	HULY	+		+	(11)	(17)
A/CL	Cadmium chloride	10108-64-2	−	CHL	+	+	−	Several	+		+	(11)	(17)
			−	Several	+		−	CHO	+			(10)	(10)
			+	HULY	−							(10)	
CL	Cadmium sulphate	10124-36-4	−	V79	+	+	−	HY	+		+	(7)	(17)
A	Carbendazim (methyl-2-benzimidazole carbamate)	10605-21-7	−	V79	+	+		HULY	−		−	(7)	(10)
				Several	+							(10)	
A	Carbon tetrachloride	56-23-5	−	H2E1, MCL-5	+	+	−/+	CHO	−		−	(25)	NTP database
			−	AHH-1	−							(25)	
CL	Catechol	120-80-9	−	CHL	+	+	−	SHE	+		+	(11)	(26)
A/CL	Chloral hydrate	302-17-0	−	V79	+	+	−	L5178Y	+		+	(27)	(28)
			−	Several	+							(10)	

Table III. *Continued*

Class	Chemical	CAS No.	S9 mix	Cells, MNT	MNT	Overall MNT	S9	Cells, CAT	CAT		Overall CAT	References, MNT	References, CAT
									SA	NA			
			+	HULY	−							(10)	
A	Chlordane	57-74-9	−	Beluga whale skin fibroblasts	+	+	−/+	CHO	−		−	(29)	NTP database
CL	*m*-Chloroaniline	108-42-9	−	CHL	Weak +	+	−	CHL	+	+	+	(11)	Concurrent test[a]
CL	*p*-Chloroaniline	106-47-8	+	CHL	−	−	+	CHL	+	−	+	(11)	(17)
CL	2-Chloro-*n*-butyric acid	4170-24-5	−	CHL	+	+	−	CHL	+	−	+	(11)	Concurrent test[a]
CL	2-(Chloromethyl)pyridine.HCl	6959-47-3	+	CHO	+	+	−/+	CHO	+		+	(22)	NTP database
CL	2-Chloro-4-nitroaniline	121-87-9	−	CHL	−	−	−	HULY	+		+	(11)	(30)
CL	Chromic acetate	1066-30-4	−	CHL	Weak +	+	−	HULY, CHO	+	−	+	(11)	(17)
CL	Chromic chloride	10025-73-7	−	CHL	Weak +	+	−	HULY	+	−	i	(11)	(17)
							−	SH fibroblasts	−				(17)
CL	Ciprofloxacin	86393-32-0	−	CHO	+	+	−/+	CHO	+		+	(9)	(9)
CL	Ciprofibrate	52214-84-3	−	Rat hepatocytes	+	+	−	Rat hepatocytes	+		+	(31)	(32)
NG/C	Clofibrate	637-07-0	−	Rat hepatocytes	−	−	−	CHL	+		i	(33)	(17)
							−	SHE	−				(34)
A/CL	Colchicine	64-86-8	−	CHL	+	+		Not given	+	+	+	(11)	(35)
			−	Several	+		−	CHO	+			(10)	(10)
CL	Coumarin	91-64-5	−	Rat hepatocytes	−	−	+	CHO	+		+	(31)	(36)
CL	CP67804	None given	−	CHO	+	+	−	CHO	+		+	(9)	(9)
CL/A	Cyclophosphamide hydrated and anhydrous	6055-19-2	+	CHL	+	+	+	CHL	+	−	+	(11)	Concurrent test[a]
		50-18-0	+	V79	+		−/+	Several	+			(7)	(17)
			−	Several	i		+	Several	+			(10)	(10)
			+	Several	+							(10)	
CL	Cytosine arabinoside	147-94-4	−	V79	+	+	−	Several	+		+	(7)	(17)
NG/C	Dichlorodiphenyltrichloroethene	50-29-3	−	Beluga whale skin fibroblasts	+	+	−	B14F28	+		i	(29)	(17)
							−	V79	−				(17)
A	*N*-deacetyl-*N*-methylcolchicine (colcemid)	477-30-5	−	V79	+	+	−	CHL	−	+	+[c]	(7)	(37)
			−	CHL	+				+[d]			(37)	(36)
NG	3,5-Diaminobenzoic acid	535-87-5	+	CHL	−	−		Not given	−	−	−	(11)	(11)
CL/NC	2,6-Diaminotoluene.2HCl	15481-70-6	−/+	CHO	+	+	−	CHO	+		+	(23)	NTP database
							−	CHO	+				(38)
A	Diazepam	439-14-5	−	V79	+	+	−/+	Several	−		−	(27)	(17)
			−	V79	+		−	Several	−			(7)	(10)
			−	Several	+							(10)	
CL/NC	Diazinon	333-41-5	−	HULY	E	E	+	CHL	+		+	(39)	(17)
CL	Dichloroacetic acid	79-43-6	−	L5178Y	−	−	−	L5178Y	+		+	(28)	(28)
NG/C	1,4-Dichlorobenzene	106-46-7	−	Rat hepatocytes	+	i	−/+	CHO	−		−	(40)	NTP database
			−	Human hepatocytes	−							(40)	
CL/A	1,2-Dichloroethane	107-06-2	−	MCL-5, AHH-1, H2E1	+	+	+	CHO	+		+	(25)	NTP database
CL/A	1,2-Dichloropropane	78-87-5	−	MCL-5, AHH-1, H2E1	+	+	−/+	CHO	+		+	(25)	NTP database
NG/C	Di(2-ethylhexyl)adipate	103-23-1	−	Rat hepatocytes	−	−	−	CHO	E		E	(31)	NTP database
NG/C	Di(2-ethylhexyl)phthalate	117-81-7	−	Rat hepatocytes	−	−	−/+	Several	−		−	(31)	(17)
			+/−	V79	−							(7)	
A	Diethylstilbestrol	56-53-1	−	V79	+	+	−/+	Several	+	+	+	(7)	(17)
			−	Several	+							(10)	
			+	Several	+		−	Several	i			(10)	(10)
	Diethylstilbestrol (*cis* and *trans*)	6898-97-1	−	CHL	+		+	Several	i			(11)	(10)
CL/NC	Dimethoate [AKA phosphorodithioic acid,*o*,*o*-dimethyl ester,*S*-ester with 2-mercapto-*N*-methylacetamide]	60-51-5	−	HULY	E	E	−	CHL	+		+	(39)	(17)

Table III. *Continued*

Class	Chemical	CAS No.	S9 mix	Cells, MNT	MNT	Overall MNT	S9	Cells, CAT	CAT		Overall CAT	References, MNT	References, CAT
									SA	NA			
CL/A	*N*,*N*-dimethylaniline	121-69-7	–	V79	+	+	+	CHO	+		+	(41)	(42)
CL	7,12-Dimethylbenz[*a*]anthracene	57-97-6	+	CHL	+	+	+	CHL	+	–	+	(11)	Concurrent test[a]
			+	V79	+		–/+	Several	+			(7)	(17)
			–	SHE	+		–/+	CHL	E			(10)	(10)
			+	Several	+		+	Several	+			(10)	(10)
CL	Dimethylnitrosamine	62-75-9	+	CHL	+	+	+	CHL	+	–	+	(10)	Concurrent test[a]
NG/NC	Dimethyl terephthalate	120-61-6	Not given	HULY	–	–	–/+	CHO	–		–	(43)	(44)
									–				(43)
A	Econazole	27220-47-9	–	Several	–	i			No data	–	i	(10)	(35)
			–	Luc-2	E							(45)	
			–	Cl-1	+							(46)	
CL	Enrofloxacin	93106-60-6	–	CHO	+	+	–/+	CHO	+		+	(9)	(9)
A	17-B-estradiol	50-28-2	–	HULY	+	+	–/+	HULY	–	+	+[c]	(47)	(17)
CL	2-Ethoxybenzamide	938-73-8	–	CHL	+	+	–	CHL	+	+	+	(11)	Concurrent test[a]
CL/A	Ethyl methanesulphonate	62-05-0	–	CHL	+	+	–	Several	+	–	+	(11)	(17)
			–	V79	+		–	Several	+			(7)	(10)
			–	Several	+							(10)	
CL	*N*-Ethyl-*N*′-nitro-*N*-nitrosoguanidine	4245-77-6	–	CHL	+	+	–	CHL	+	–	+	(11)	(17)
CL	*N*-Ethyl-*N*-nitrosourea	759-73-9	–	CHL	Weak +	+	–	Several	+	–	+	(11)	(17)
CL	5-Fluorouracil	51-21-8	–	CHL	+	+	–	CHO/CHL	+	–	+	(11)	(17)
			–	V79	E		–	Several	+			(7)	(10)
			–	Several	+							(10)	
			+	CHL	+							(10)	
CL	Fumonisin B1	116355-83-0	–	Rat hepatocytes	+	+	–	Rat hepatocytes	+		+	(48)	(48)
A	Griseofulvin	126-07-8	–	Rat hepatocytes	+	+	–	HULY, EUE	+	+	+	(31)	(17)
			–	V79	+		–	HULY	E			(7)	(10)
			–	Several	+							(10)	
?	Hexachlorobenzene	118-74-1	–	Rat hepatocytes	+	+	–	CHL	TC		TC	(40)	(17)
			–	Human hepatocytes	+							(40)	
NG/C	Hexachloroethane	67-72-1	–	MCL-5, AHH-1, H2E1	–	–			–		–	(25)	NTP database
CL	Hydrogen peroxide	7722-84-1	–	CHL	+	+	–	Several	+	–	+	(11)	(17)
A/CL	Hydroquinone	123-31-9	–	CHL	+	+	–	CHL	+		+	(11)	Concurrent test[a]
			–	V79	+		–	CHO	E			(7)	(10)
			–	Several	+		+	CHO	+			(10)	(10)
			+	HULY	–							(10)	
CL/NC	Malathion	121-75-5	–	HULY	+	+	+	CHO	+		+	(49)	(36)
NG/NC	Maleic hydrazide	123-33-1	–/+	HULY	–	–	–	HULY	E		E	(50)	(50)
CL	6-Mercaptopurine	50-44-2	–	CHL	+	+	–	HULY, CHO, CHL	+	–	+	(11)	(17)
CL	Mercuric chloride	7487-94-7	–	HULY	+	i	–	HULY	+		+	(51)	(51)
			–	CHL	–		–	FM3A	TC	–		(11)	(17)
CL/NC	Methotrexate	59-05-2	–	V79	i	i	–/+	CHO, A(T1)CL-3	+		+	(7)	(17)
CL	3-Methylcholanthrene	56-49-5	–	MCL-5	+	+	+	CHL,	+		+	(20)	(17)
			+	CHO	–		–	RL1	+			(10)	(17)
			+	CHL, L5178Y	+		–	CHL	–			(10)	(10)
							+	CHL, L5178Y	+				(10)
G[e]/C	4,4-Methylenebis(2-chloroaniline)	101-14-4	–	CHL	Weak +	+	–	CHL	–	+	+[c]	(11)	Concurrent test[a]
A	Methylmercury chloride	115-09-3	–	CHL	+	+	–	HULY	+	+	+	(11)	(52)
CL	Methyl methanesulphonate	66-27-3	–	CHL	+	+	–	Several	+	–	+	(11)	(17)
			–	Several	+		–	Several	+			(10)	(10)
G[e]	2-Methyl-4-nitroaniline	99-52-5	–	CHL	–	–	–	CHL	–	+	+[c]	(11)	Concurrent test[a]
CL/A	*N*-Methyl-*N*′-nitro-*N*-nitrosoguanidine	70-25-7	–	CHL	+	+	–	Several	+	–	+	(11)	(17)

Table III. *Continued*

Class	Chemical	CAS No.	S9 mix	Cells, MNT	MNT	Overall MNT	S9	Cells, CAT	CAT		Overall CAT	References, MNT	References, CAT
									SA	NA			
			−	Several	+		−	Several	+			(10)	(10)
CL	1-Methyl-1-nitrosourea	684-93-5	−	Several	+	+	−	CHO	+		+	(10)	(10)
NG	Methylurea	598-50-5	−	V79	−	−	−	CHL, Don	TC		TC	(7)	(16)
CL	Mitomycin C	50-07-7	−	CHL	+	+	−	Several	+	−	+	(11)	(17)
			−	V79	+		−	Several	+			(7)	(10)
			−	Several	+							(10)	
CL	Monocrotaline	315-22-0	−	Rat hepatocytes	+	+	+	CHO	+		+	(31)	(17)
							−	Rat fibroblasts	−				(17)
NG/C	Nafenopin	3771-19-5	−	Rat hepatocytes	−	−	−	Rat hepatocytes	+		+	(31)	(32)
NG/C	Nalidixic acid	389-08-2	−	CHO	−	−	−/+	CHO	−		−	(53)	NTP database
			−	CHO	−		−	CHO	−			(9)	(9)
CL	b-Naphthoquinoline	85-02-9	−	CHL	+	+	−/+	CHL	+		+	(11)	(17)
CL/NC	*N*-(1-naphthyl)ethylenediamine. 2HCl [AKA PL-89779]	1465-25-4	−/+	CHO	−	−	+	CHO	+		+	(22)	NTP database
CL	Neocarcinostatin	9014-02-2	−	Several	+	+	−	Several	+		+	(10)	(10)
CL	Nickel acetate	373-02-4	−	CHL	+	+	−	FM3A	+		+	(11)	(17)
CL	Nickel chloride	7718-54-9	−	CHL	+	+	−	FM3A	+		+	(11)	(17)
A	Nitrilotriacetic acid	139-13-9	−	Cl-1	+	+	−/+	CHO	−		−	(54)	(42)
E	*o*-Nitroaniline	88-74-4	−	CHL	−	−	−/+	CHO			i	(11)	(55)
CL/NC	4-Nitroanthranilic acid	619-17-0	−/+	CHO	−	−	+	CHO	+		+	(22)	NTP database
CL	2-Nitrofluorene	607-57-8	−	CHL	Weak +	+	+	CHL	+	−	+	(11)	(56)
			−	Several	+		−	CHL	E			(10)	(10)
			+	CHL	+		+	CHL	+			(10)	(10)
CL/NC	4-Nitro-*o*-phenylenediamine	99-56-9	−/+	CHO	−	−	−	CHMP/E	+		+	(22)	(57)
G[e]/NC	3-Nitropropionic acid	504-88-1	−/+	CHO	E	E	−	CHO	E		E	(22)	NTP database
CL	4-nitroquinoline-*N*-oxide	56-57-5	−	L5178Y	+	+	−	Several	+		+	(20)	(17)
CL	*N*-nitrosodiethylamine (diethylnitrosamine)	55-18-5	−	Rat hepatocytes	+	+	+	CHL, CHO	+		+	(31)	(17)
NG/C	*N*-nitrosodiphenylamine	86-30-6	−/+	CHO	−	−	−/+	CHO	−		−	(58)	(44)
CL	*m*-Nitrotoluene	99-08-1	−	CHL	Weak +	+	−	CHL	TC	+	+	(11)	(17)
							−	HULY	+				(29)
CL	*o*-Nitrotoluene	88-72-2	−	CHL	+	+	−	CHL	TC	+	+	(11)	(17)
							−	HULY	+				(30)
CL	*p*-Nitrotoluene	99-99-0	−	CHL	−	−	−	CHL	TC	+	+	(11)	(17)
							−	HULY	+				(30)
CL	Norfloxacin		−	CHO	−	−	−/+	CHO	−		−	(9)	(9)
CL	Ofloxacin	85344-55-4	−	CHO	+	+	−	CHO	+		+	(9)	(9)
A	Oxazepam	604-75-1	−	SHE, AFFL, L5178Y	+	+	−/+	CHO	−		−	(59)	NTP database
CL	Phenacetin	62-44-2	+	CHL	−	−	+	CHL	+		+	(11)	(17)
G[e]	Phenobarbital	50-06-6	−	Rat hepatocytes	−	−	−/+	CHO, CH1-L	+		+	(31)	(17)
CL/NC	Phenol	108-95-2	+	CHL	Weak +	+	+	CHO	+		+	(11)	(60)
			−/+	CHO	+		−	CHO	E			(23)	(10)
			−/+	CHO	+							(19)	
			−	HULY	+							(61)	
CL	Phenolphthalein	77-09-8	−	MCL-5	+	+	+	CHO	+		+	(62)	NTP database
CL	*m*-Phenylenediamine	108-45-2	−	CHL	+	+	−	CHL	+	−	+	(11)	Concurrent test[a]
								CHL	+				(17)
CL	*p*-Phenylenediamine	106-50-3	+	CHL	+	+	+	CHL	+	−	+	(11)	(17)
CL/NC	p-Phenylenediamine.2HCl	624-18-0	−	CHO	+	+	−/+	CHO	+		+	(22)	NTP database
CL	Potassium bromate	7758-01-2	−	CHL	+	+	−	CHL	+	−	+	(11)	Concurrent test[a]
							−	CHL	+				(17)
NG/NC	Promethazine.HCl	58-33-3	+	V79	E	E	−/+	CHO	−		−	(63)	(36)
NG/NC	Pyrene	129-00-0	−	V79	−	−	−/+	Several	−		−	(7)	(17)

Table III. *Continued*

Class	Chemical	CAS No.	S9 mix	Cells, MNT	MNT	Overall MNT	S9	Cells, CAT	CAT		Overall CAT	References, MNT	References, CAT
									SA	NA			
			−	SHE, HepG2	−		−	CHL, CHO, DON	−			(10)	(10)
			+	CHO	−		+	CHO, CHL	−			(10)	(10)
			+	L5178Y	−							(18)	
A/CL	Pyrimethamine	58-14-0	−	AHH-1	+	+	−	HULY	+		+	(25)	(64)
			−	CHL	+							(10)	
			−	V79	i		−	CHL	+			(10)	(10)
			+/−	HULY	−		+	CHL	−			(10)	(10)
?	Retinol acetate	127-47-9	−	V79	+	+	−	CHL	TC		TC (−)	(7)	(17)
A/NC	Rotenone	83-79-4	−/+	HULY	+	+	−/+	CHO	−		+[c]	(5)	(65)
							−/+	HULY	−				(66)
							−	CHL	−	+			(67)
NG/C	Safrole	94-59-7	+	L5178Y	+	+	+	CHL, CHO	+		+	(18)	(17)
							−	Several	−				(17)
NG[d]	Sodium chloride	7647-14-5	−	CHL	−[d]	−	−	HULY	TC		TC	(11)	(17)
			−	16 MA	+[d]		−	16 MA	+[d]			(68)	(68)
NG/C	Tetrachloroethylene	127-18-4	−	CHL	−	−	−	CHL	−	−	−	(11)	Concurrent test[a]
NG/C	12-*o*-Tetradecanoylphorbol-13-acetate	16561-29-8	−	CHL	−	−	−	CHL	−	−	i	(11)	Concurrent test[a]
							−	HULY, Mouse PEC	+	+			(17)
							−	CHO, CHL	−				(17)
A	Thiabendazole	148-79-8	−	V79	+	+	−	CHO, CHL	TC	+	+[c]	(7)	(17)
			−	V79	+							(10)	
			−/+	HULY	−							(10)	
NG/C	Titanium dioxide	13463-67-7	−/+	CHO	−	−	−/+	CHO	−		−	(23)	(60)
NG/C	Toluene	108-88-3	+/−	V79	i	i	−/+	CHO	−		−	(7)	NTP database
CL	Triamterene	396-01-0	−	Don-6	+	+	−	CHL	+		+	(69)	(17)
							+	CHL	−				(17)
A	1,1,2-Trichloroethane	79-00-5	−	H2E1, MCL-5	+	+	−/+	CHO	+		+	(25)	NTP database
			−	AHH-1	−							(25)	
A	1,1,2-Trichloroethylene (with and without epichlorohydrin)	79-01-6	−	MCL-5	+	i	−	CHL	−	−	−	(25)	(24)
			−	AHH-1	−		−/+	CHO	−			(25)	(36)
			−	V79	−				−			(7)	
CL	1,2,3-Trichloropropane	96-18-4	−	MCL-5	+	i	+	CHO	+		+	(25)	NTP database
			−	H2E1, AHH-1	−							(25)	
NG/NC	Triphenyltin hydroxide	76-87-9	+	CHO	+	+	−/+	CHO	−		−	(22)	NTP database
NG[d]	Urethane	51-79-6	+	CHL	−	−	−	CHL	−[d]	−	−	(11)	(5)
			−	V79	−							(7)	
A	Vinblastine	143-67-9	−	CHL	+	+	−	CHL	−	+	+[c]	(11)	Concurrent test[a]
			−	V79	+		−	Don	+			(7)	(70)
A	Vincristine sulphate	5722-7	−	Several	+	+	−	CHL	+		+	(10)	(10)

CL, clastogens; A, aneugens; NG, non-genotoxic or equivocal; NC, non carcinogen; C, carcinogen; MNT, *in vitro* MNT; CAT, *in vitro* CAT; SA, structural aberrations; NA, numerical aberrations and TC, technically compromised; NA was not always available; E, equivocal; i, inconclusive. Data on compounds that were reported in more than one publication (e.g. Ishidate and Miller) were reported only once. German trial results: re-evaluation of the VMT. Cell type is reported only when available.

[a]Chromosomal aberration tests were performed concomitantly under the same experimental condition.

[b]Lack of clear genotoxicity may be due to unusual *in vivo* metabolism in tumour target tissue.

[c]The overall call takes into consideration the numerical berration data.

[d]Positive results obtained only at the extremely high concentrations (>10 mM) were excluded.

[e]More clearly genotoxic in systems other than those detecting A or CL.

Table IV. Concordance *in vitro* MNT/*in vitro* CAT for all compounds

MNT	CAT		
	+	−	Total
+	83	9[a]	92
−	10	11	21
Total	93	20	113

[a]Carbendazim, carbon tetrachloride, chlordane, diazepam, nitriloacetic acid and oxazepam (all known as pure aneugenic compounds) were correctly negative in the *in vitro* CAT but positive in the *in vitro* MNT.

Table V. Concordance for compounds classified as clastogenic (CL)

MNT	CAT		
	+	−	Total
+	61 (86%)	0	61
−	9[a] (13%)	1[b] (1%)	10
Total	70	1	71

[a]*p*-Chloroaniline (not convincingly clastogenic), 2-chloro-4-nitroaniline, coumarin, dichloroacetic acid, 2-methyl-4-nitroaniline, *N*-(1-naphthyl)ethylenediamine.2HCl, 4-nitroanthranilic acid, 4-nitro-*o*-phenylenediamine and phenacetin.
[b]Norfloxacin.

Table VI. Concordance for compounds classified as aneugenic (A) or aneugenic/clastogenic (A/CL)

MNT	CAT		
	+	−	Total
+	21 (78%)	6[a] (22%)	27
−	0	0	0
Total	21	6	27

[a]Carbendazim, carbon tetrachloride, chlordane, diazepam, nitrilotriacetic acid, oxazepam—remark: all six compounds classified as pure aneugens (A).

Table VII. Concordance for compounds classified as non-genotoxic (NG)

MNT	CAT		
	+	−	Total
+	1[a] (6.5%)	3[b] (20%)	4
−	1[c] (6.5%)	10 (67%)	11
Total	2	13	15

[a]Safrole.
[b]l-Ascorbic acid, *n*-butyl-*N*-(3-carboxypropyl)nitrosamine and triphenyltin hydroxide.
[c]Nafenopin.

principles on test validity and does fulfil the criteria for a successful validation (Table IX). Therefore, the VMT concluded that the *in vitro* MNT can be recommended as an alternative/replacement for the *in vitro* CAT for genotoxicity testing (hazard identification). After a thorough peer review, this conclusion was unanimously endorsed by all members of the ESAC.

Table VIII. Summary of the concordance evaluations

	No compounds	Concordance %	Sensitivity %	Specificity %
All compounds	113	83.2	89.2	55.0
Clastogens[a]	71	87.3	_	_
Aneugens[b]	27	77.8	_	_
Non-genotoxic[c]	15	73.3	_	_
German ring trial (7)	20	85.0	92.9	66.7
Miller *et al.* (9)	54	83.3	95	76.5
Japanese ring trial (11)	62	82.3	91.5	53.3
GUM Working group (10)	27	88.9	100	25
Kirkland *et al.* (12)	125	80.8	87.0	56.0

[a]Norfloxacin was negative in both assays whereas chloroaniline (not convincingly clastogenic), 2-chloro-4-nitroaniline, coumarin, dichloroacetic acid, 2-methyl-4-nitroaniline, *N*-(1-naphthyl)ethylenediamine2HCl, 4-nitroanthranilic acid, 4-nitro-*o*-phenylenediamine and phenacetin were negative in the *in vitro* MNT but positive in the *in vitro* CAT.
[b]Carbendazim, carbon tetrachloride, chlordane, diazepam, nitrilotriacetic acid and oxazepam (all known as pure aneugenic compounds) were negative in the *in vitro* CAT but positive in the *in vitro* MNT.
[c]Nafenopin was negative in the *in vitro* MN but positive in the *in vitro* CAT, l-ascorbic acid, *n*-butyl-*N*-(3-carboxypropyl)nitrosamine and triphenyltin hydroxide were positive in the *in vitro* MNT but negative in the *in vitro* CAT whereas only safrole was positive in both assays.

This evaluation demonstrated that the *in vitro* MNT has the potential to reliably identify clastogens and to enhance the basic battery of *in vitro* tests by its capability to detect aneugens. Most of the established aneugens (defined as *in vivo* aneugens) have been tested with the *in vitro* MNT and scored positive. 'Pure' aneugens were only positive in the *in vitro* MNT and not in the standard *in vitro* CAT, if polyploidy and chromosome count were not considered.

Nine chemicals tested were found to be positive only in the *in vitro* CAT and not in the *in vitro* MNT. It is well known that the *in vitro* CAT is prone to clastogenicity induction at high toxicity levels. The effect has been discussed by several experts to be irrelevant for the *in vivo* situation. One could now speculate that the *in vitro* MNT is less prone to such non-predictive positive effects. However, comparison to a 'gold standard' always has the drawback that the assumption is made that the results of this standard are 100% correct. Comparison to carcinogenicity, for example, has the same limitations due to the fact that carcinogenicity studies are rarely repeated and the result obtained is always taken as 100% correct for comparisons.

One of the main difficulties in a retrospective validation study is the lack of a standardized protocol. As in the case of the *in vitro* MNT evaluated in this retrospective validation, the scopes of the available studies used were very different (7,8). Based on these differences, the high reproducibility and concordance found for the *in vitro* MNT underlines the robustness of the test.

The outcome of this evaluation is a very important contribution to the ECVAM validation process because for the first time a test has been validated based on existing data only (retrospective validation) and will, therefore, lay the ground for future retrospective validation studies. This approach may be instrumental in the validation of alternative methods that will contribute in finding more effective ways of testing and assessing the toxicological and health impacts of chemicals under the new European chemicals legislation [Regulation Evaluation Authorisation of Chemicals (REACH)].

Table IX. Conclusions of the VMT for the different modules (5)

		Conclusion
Test definition	√	Clear definitions of the scientific basis, description of the end points and the mechanistic basis; protocol requirements available.
Within-laboratory reproducibility	√	The within-laboratory reproducibility was in an acceptable range (94–100% assessed per treatment independent from cell model; 97–100% reproducibility per cell line, independent from treatment)
Transferability	√	Test method can be easily transferred and no extraordinary facilities are required. Overall, the successful transferability of the *in vitro* MNT is demonstrated by the satisfactory results for the between-laboratory variability from the two studies evaluated.
Between-laboratory reproducibility	√	The between-laboratory reproducibility assessed per treatment, independent from cell line varied between 86 and 100%. The between-laboratory reproducibility assessed per cell model, independent from treatment varies from 79 to 100%.
Predictive capacity (concordance)	√	The concordance between *in vitro* MNT and *in vitro* CAT ranges from 80.8 to 88.9% in the different studies. The general concordance for all compounds is 83.2%, the concordance for clastogens is 87.3% and the concordance for aneugens is 77.8%. However, all compounds known to induce aneuploidy were detected by the MNT *in vitro*.
Applicability domain	√	Genotoxicity (structural and numerical chromosome aberration); all chemical classes; potential to be used in screening strategy for genotoxicity evaluation (regulatory use).

The successful validation of the *in vitro* MNT and its endorsement by the independent ESAC has led to European Union regulatory acceptance and to the quick integration in the genotoxicity testing requirements foreseen in the REACH legislation. Currently, the ICH (International Conference on Harmonisation for pharmaceuticals) is also considering recommending the *in vitro* MNT as an alternative to the *in vitro* CAT and mouse lymphoma TK assay for the detection of clastogenic/aneugenic potential based on the 'validation status' received by ECVAM. Furthermore, the formal (retrospective) validation by ECVAM should support the finalization of the TG and its regulatory acceptance by the OECD. OECD acceptance of the *in vitro* MNT will lead to its widespread international application.

Supplementary data

Supplementary material is available at *Mutagenesis* Online.

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