ECVAM retrospective validation of \textit{in vitro} micronucleus test (MNT)

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In the past decade several studies comparing the \textit{in vitro} chromosome aberration test (CAT) and the \textit{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 \textit{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 \textit{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 \textit{in vitro} MNT as alternative to the standard \textit{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. \textit{et al.} (2000) \textit{In-vitro} micronucleus assay with Chinese hamster V79 cells: results of a collaborative study with 26 chemicals. \textit{Mutat. Res.}, 468, 137–163, and SFTG International Collaborative Study, Lorge, E., Thybaud, V., Aardema, M., Oliver, J., Watakase, A., Lorenzo, G. and Marzin, D. (2006) SFTG International Collaborative Study on in-vitro micronucleus test I. General conditions and overall conclusions of the study. \textit{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 \textit{in vitro} MNT and the \textit{in vitro} CAT, additional published data were considered. Based on this retrospective validation, the ECVAM Validation Management Team concluded that the \textit{in vitro} MNT is reliable and relevant and can therefore be used as an alternative method to the \textit{in vitro} CAT. Following peer review, these conclusions were formally endorsed by the ECVAM Scientific Advisory Committee.

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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 protocol, concentrations up to 70% 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 cyclohexalin 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 magnitude 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** | **Recovery** | **S** | **S** | **L** | **L** | **S** | **S** | **L** | **L** | **S** | **S** | **L** | **S + S9** | **N** | **S** |
| HL | - | 4:4, 100% | 6:6, 100% | 8:8, 100% | 7:7 100% | 6:6, 100% | - | - | 21:21 100% |
| L5178Y | 5:5, 100% | 6:6, 100% | 5:5, 100% | 6:6, 100% | 5:5, 100% | 6:6, 100% | - | - | 30:30 100% |
| CHL | 8:8, 100% | 11:12, 92% | 9:9, 100% | 10:10, 100% | 12:12, 100% | 20:20 100% | - | - | 57:57 100% |
| CHO | 7:7, 100% | 5:5, 100% | 7:7, 100% | 27:27, 100% | 26:26, 96% | - | - | - | 27:27, 100% |
| V79 | 19:19, 100% | 21:21, 95% | 17:18, 94% | 27:27, 100% | 12:12, 100% | - | - | - | 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)

| Treatment | CAT | Without CB | SFTG ring trial | German ring trial |
|-----------|-----|------------|-----------------|------------------|
|           |     | Without CB | With CB         |                  |
|           |     |            |                 |                  |
| Recovery  | S   | L          | L               | S                |
| HL        | S   | –          | –               | –                |
| L5178Y    | S   | 1:1, 100%  | 3:3, 100%       | 3:3, 100%        |
| CHO       | L   | –          | –               | –                |
| CHO       | S   | 4:4, 100%  | 4:4, 100%       | 4:4, 100%        |
| V79       | L   | –          | –               | –                |
|           | S   | 11:11, 100%| 11:11, 100%     | 11:11, 100%      |

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 aneugen was 88.5%.

Moreover, to allow a concordance analysis for each chemical class, all compounds were classified in the following classes: clastogens, aneugen and non-genotoxic (Tables V–VII). The concordance between in vitro MNT and in vitro CAT is 77.8% for clastogens, 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
| Class | Chemical | CAS No. | S9 mix | Cells, MNT | MNT Overall | S9 Cells, CAT | CAT Overall | References, MNT | References, CAT |
|-------|----------|---------|--------|------------|-------------|---------------|-------------|----------------|-----------------|
| CL    | Acetaminophen | 103-90-2 | − | CHL | + + + − | Several | + − + | (11) | (17) |
| CL    | Acetyl salicylic acid (aspirin) | 50-78-2 | − | CHL | Weak + + + − | CHL | + + − | (11) | (17) |
| CL    | 2-Acetylaminofluorene | 53-96-3 | + | Several | + + − | RL1 | + + | (10) | (17) |
|       |           |         | + | CHL | + + | − | − | − | − |
|       |           |         | + | V79 | + | − | − | − | − |
| CL    | Actinomycin D | 50-76-0 | −/− | CHO | + + − | Several | + + − | (19) | (17) |
| CL    | Adriamycin | 25316-40-9 | − | Several | + + + − | Several | + + | (10) | (10) |
| CL    | Aflatoxin B1 | 53-96-3 | + | MCL-5 | + + | V79 | + + | (20) | (17) |
|       |           |         | + | Several | + + | − | − | − | − |
|       |           |         | + | HULY | + + | HULY | + + | (10) | (10) |
| CL    | 2-Aminoanthracene | 613-13-8 | + | Several | + + | CHO | E + E | (10) | (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 | + + | CHO | E + E | (10) | (10) |
| CL    | 2-Amino-3-methyl-9H-pyrido-[2, 3-b] indole acetate | 63170-60-5 | + | CHL | + + | Not available | + + | (11) | (21) |
| CL    | m-Annaquinine | 54301-15-4 | − | Several | + + + − | Several | + + | (10) | (10) |
| CL    | Aniline | 62-53-3 | + | CHL | + + + | CHO | E + E | (11) | (17) |
| CL    | o-Antiherbamic acid | 118-92-3 | + | CHO | + + − | CHO | − − | (22) | NTP database |
| NC/NG | 1-Ascorbic acid | 59-81-7 | + | CHO | + + − | CHO | − | (23) | NTP database |
| CL    | Barbitual | 57-44-3 | + | CHL | Weak + + + − | CHL | + + i | (11) | (17) |
|       |           |         | + | DON | − + | − | − | − | − |
| CL    | Benzene | 71-43-2 | + | CHL | Weak + i −/− | Several | + − − | (11) | (17) |
| CL    | Benzidine | 92-87-5 | + | MCL-5 | + + −/− | Several | + + + | (20) | (17) |
| CL    | Benzo[a]pyrene | 50-32-8 | − | CHL | Weak + + − | CHL | − − | (11) | Concurrent test* |
|       |           |         | − | SHE/3T3 | + + | − | − | − | − |
| CL    | Benzo[b]pyrene | 100-44-7 | − | Several | + + − | CHO | + + | (11) | (17) |
| NG/C  | Benzylacetate | 140-11-4 | − | V79 | i i −/− | CHO | − − | (7) | (24) |
| CL    | Bleomycin sulphate | 11056-06-7 | − | V79 | + + − | Several | + + | (7) | (17) |
|       |           |         | − | Several | + | − | − | − | − |
| NG/Cb | 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 | + + − | CHO | + − | (11) | (17) |
|       |           |         | − | Several | + | − | − | − | − |
| CL    | Cadmium sulphate | 10124-36-4 | − | V79 | + + − | HY | + + | (7) | (17) |
| A     | Carbendazim (methyl-2-benzimidazol carbamate) | 10605-21-7 | − | V79 | + + − | HULY | − − | (7) | (17) |
| A     | Carbon tetrachloride | 56-23-5 | − | H2E1, MCL-5 | + + −/− | CHO | − − | (25) | NTP database |
| CL    | Catechol | 120-80-9 | − | CHL | + + − | SHE | + + | (11) | (26) |
| A/CL  | Chloral hydrate | 302-17-0 | − | V79 | + + − | LS178Y | + + | (27) | (28) |
| Class   | Chemical                                      | CAS No. | S9 mix | Cells, MNT   | MNT Overall | S9 Overall | Cells, CAT   | CAT Overall | References, CAT |
|---------|----------------------------------------------|---------|--------|--------------|-------------|------------|--------------|-------------|-----------------|
| A CL    | m-Chloroaniline                              | 108-42-9 | −      | CHL          | Weak + + − | CHL + + − | CHO − − −    | CHO + + (11) | Concurrent test |
| A CL    | p-Chloroaniline                              | 106-47-8 | +      | + + + − CHL  | CHO + + −  | CHO + + −  | CHO + + −    | CHO + + (11) | Concurrent test |
| A CL    | 2-Chloro-n-butyric acid                      | 4174-20-5 | +      | − + − + CHL  | + + − + CHO| + + − + CHO| + + − + (11) | (17) NTP database |
| A CL    | 2-Chloro-n-pyridine.HCl                      | 6959-47-3 | +      | − + − + HULY | + + − + CHO| + + − + (22) | (17) NTP database |
| A CL    | Chronic acetate                              | 1066-30-4 | −      | − + − + HULY | + − − + (17)| + − − + (17) | (30) |
| A CL    | Chronic chloride                             | 120025-73-7 | +      | + + − + HULY | + + − + (17)| + − − + (17) | (30) |
| A CL    | Ciprofloxacin                                | 86393-32-0 | +      | + + − CHO    | + + − CHO  | + + − CHO  | + + − (9)    | (9) |
| A CL    | Ciprofibrate                                 | 52214-84-5 | −      | − + − CHO    | + + − CHO  | + + − CHO  | + + − (31)   | (32) |
| A/CL    | Colchicine                                   | 64-86-8  | −      | − + − CHO    | − + − CHO  | − + − CHO  | − + − (33)   | (34) |
| A/CL    | Coumarin                                     | 91-64-5  | −      | − + − CHO    | − + − CHO  | − + − CHO  | − + − (35)   | (35) |
| A CL/A  | Cyclophosphamid hydrated and anhydrous       | 6055-19-2 | +      | − + − CHO    | − + − CHO  | − + − CHO  | − + − (9)    | (9) |
| A CL/A  | m-Chloroformic acid                          | 50-18-0  | +      | − + − CHO    | − + − CHO  | − + − CHO  | − + − (11)   | (11) NTP database |
| A CL/A  | Cytosine arabinoside                         | 147-94-4 | −      | − + − CHO    | − + − CHO  | − + − CHO  | − + − (7)    | (7) |
| A NG/C  | Dichlorodiphenyltrichloroethene              | 50-29-3  | −      | − + − CHO    | − + − CHO  | − + − CHO  | − + − (29)   | (17) |
| A NG/C  | N-deacetyl-N-methylcolchicine (colcemid)     | 33-93-6  | −      | − + − CHO    | − + − CHO  | − + − CHO  | − + − (37)   | (37) |
| A NG/C  | 5,5-Diaminobenzoic acid                      | 477-30-5 | −      | − + − CHO    | − + − CHO  | − + − CHO  | − + − (36)   | (36) |
| A CL/NC | 2,6-Diaminotoluene.HCl                       | 15481-70-6 | −/+   | − + − CHO    | − + − CHO  | − + − CHO  | − + − (23)   | (28) |
| A CL/NC | Diazepam                                     | 439-14-5 | −      | − + − CHO    | − + − CHO  | − + − CHO  | − + − (38)   | (38) |
| A CL/NC | Diazinon                                     | 333-41-5 | −      | − + − CHO    | − + − CHO  | − + − CHO  | − + − (11)   | (11) NTP database |
| A CL/NC | Dichlorobacitracin                           | 379-43-6 | −      | − + − CHO    | − + − CHO  | − + − CHO  | − + − (27)   | (27) |
| A CL/NC | Dichloroacetic acid                          | 79-43-6  | −      | − + − CHO    | − + − CHO  | − + − CHO  | − + − (10)   | (10) NTP database |
| A CL/NC | Bis-Dichloroacetic acid                      | 106-46-7 | −      | − + − CHO    | − + − CHO  | − + − CHO  | − + − (40)   | (40) |
| A CL/NC | Di(2-ethylhexyl)chloroethene                 | 107-06-2 | −      | − + − CHO    | − + − CHO  | − + − CHO  | − + − (25)   | (25) NTP database |
| A CL/NC | Di(2-ethylhexyl)phthlate                     | 78-87-5  | −      | − + − CHO    | − + − CHO  | − + − CHO  | − + − (25)   | (25) NTP database |
| A CL/NC | Diethylstilbestrol                           | 56-53-1  | −      | − + − CHO    | − + − CHO  | − + − CHO  | − + − (31)   | (31) NTP database |
| A CL/NC | Dimethoate [AKA phosphorodithioic acid, o-o-| 6898-97-1 | −      | − + − CHO    | − + − CHO  | − + − CHO  | − + − (39)   | (39) |
| A CL/NC | Dimethoate (cis and trans)                   | 60-51-5  | −      | − + − CHO    | − + − CHO  | − + − CHO  | − + − (39)   | (39) |

References:

- A Chlordane
- CL m-Chloroaniline
- CL p-Chloroaniline
- CL 2-Chloro-n-butyric acid
- CL 2-Chloro-n-pyridine.HCl
- CL Chronic acetate
- CL Chronic chloride
- CL Ciprofloxacin
- CL Ciprofibrate
- A/CL Colchicine
- CL Coumarin
- CL Cyclophosphamid hydrated and anhydrous
- CL Cytosine arabinoside
- NG/C Dichlorodiphenyltrichloroethene
- A N-deacetyl-N-methylcolchicine (colcemid)
- NG 5,5-Diaminobenzoic acid
- CL/NC 2,6-Diaminotoluene.HCl
- A Diazepam
- CL/Diazinon
- CL Dichloroacetic acid
- NG/C 1,4-Dichlorobenzene
- CL/A 1,2-Dichloroethane
- CL/A 1,2-Dichloropropene
- NG/C Di(2-ethylhexyl)adipate
- NG/C Di(2-ethylhexyl)phthlate
- A Diethylstilbestrol
- CL/NC Dimethoate [AKA phosphorodithioic acid, o-o-dimethyl ester, o-ester with 2-mercapto-N-methylacetamide]
| Class | Chemical                          | CAS No. | S9 mix | Cells, MNT | Overall MNT | S9 Cells, CAT | CAT SA | Overall CAT | References, MNT | References, CAT |
|-------|----------------------------------|---------|--------|------------|-------------|---------------|--------|-------------|-----------------|-----------------|
| CL/A  | N,N-dimethylaniline              | 121-69-7| –      | V79        | +           | +             | CHO    | +           | (41) (42)       |                 |
| CL    | 7,12-Dimethylbenz(α)anthracene  | 57-97-6 | +      | CHL        | +           | +             | CHL    | +           | (11) Concurrent test* |
|       |                                  |         | +      | V79        | +           | +             | CHL    | +           |                 |
|       |                                  |         | +      | SHE        | +           | –/+           |        |              |                 |
| CL    | Dimethylnitrosamine              | 62-75-9 | +      | CHL        | +           | +             | CHL    | +           | (10) Concurrent test* |
| NG/NC | Dimethyl terephthalate           | 120-01-6| Not given | HULY     | –           | –             | CHO    | –           | –               | (43) (44)       |
| A     | Econazole                        | 27220-47-9| –      | Several   | –           | i             |        | No data     | –               | (10) (35)       |
|       |                                  |         | –      | Luc-2      |             | E             |        |             |                 |
|       |                                  |         | –      | Cl-1       |             |               |        |             |                 |
| CL    | Enrofloxacin                     | 93106-60-6| –      | CHO        | +           | +             | CHO    | +           | (9) (9)         |
| A     | 17-B-estradiol                   | 50-28-2 | –      | HULY       | +           | +             | HULY   | –           | (47) (17)       |
| CL    | 2-Ethoxybenzamide                | 938-73-8| –      | CHL        | +           | +             | CHL    | +           | (11) Concurrent test* |
| CL/A  | Ethyl methanesulphonate          | 62-05-0 | –      | CHL        | +           | –             | Several| +           | (7) (10)        |
|       |                                  |         | –      | V79        | +           | –             | Several|             |                 |
| 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)       |
| A     | Griseofulvin                     | 116355-83-0| –      | Rat hepatocytes | +         | +             | Rat hepatocytes | +         | (48) (48)       |
| CL    | Fumonisin B1                     | 126-07-8 | –      | Rat hepatocytes | +         | +             | HULY   | +           | (31) (17)       |
|       |                                  |         | –      | V79        | +           | –             |        | HULY         | (7) (10)        |
|       |                                  |         | –      | V79        | +           | –             |        |             |                 |
| ?     | Hexachlorobenzene                | 118-74-1| –      | Several   | +           | –             | CHL    | +           | (10)            |
| NG/C  | Hexachloroethane                 | 67-72-1 | –      | Several   | +           | –             | CHL    | +           | (10) (40)       |
| A/CL  | Hydrogen peroxide                | 7723-84-1| –      | CHL        | +           | –             | Several| +           | (11) Concurrent test* |
|       | Hydroquinone                     | 123-31-9| –      | CHL        | +           | +             | CHL    | +           | (11)            |
|       |                                  |         | –      | V79        | +           | –             | CHL    | +           | (11) Concurrent test* |
|       |                                  |         | +      | V79        | +           | –             | CHL    | +           | (10) (10)       |
| CL/NC | Malathion                        | 121-75-5| –      | HULY       | +           | +             | CHO    | +           | (49) (36)       |
| NG/NC | Maleic hydrazide                 | 123-33-1| –/+    | HULY       | –           | –             |        |              |                 |
| CL    | 6-Mercaptopyrimidine             | 50-44-2 | –      | CHL        | +           | +             | HULY   | CHO, CHL    | (11) Concurrent test* |
| CL    | Mercarc chloride                 | 7487-94-7| –      | HULY       | +           | i             |        | +           | (51) (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      | +           | +             | RL1    | +           | (10) (17)       |
|       |                                  |         | +      | CHO        | –           | –             | CHL    | +           | (10) (10)       |
|       |                                  |         | +      | CHL        | –           | –             | CHL, L5178Y| +           |                 |
|       |                                  |         | +      | CHL, L5178Y| –           | –             |        |             |                 |
| G/C   | 4,4'-Methylenebis(2-chloroaniline)| 101-14-4| –      | CHL        | Weak        | +             | CHL    | –           | +               |
| A     | Methylnitrosurea                 | 115-09-3| –      | CHL        | +           | +             | HULY   | +           | (11) Concurrent test* |
| CL    | Methyl methanesulphonate         | 66-27-3 | –      | CHL        | +           | –             | Several| +           | (11) Concurrent test* |
|       |                                  |         | +      | Several   | +           | –             |        |             |                 |
| G'    | 2-Methyl-4-nitroaniline          | 99-52-5 | –      | CHL        | –           | –             | CHL    | +           | +               |
| CL/A  | N-Methyl-N′-nitro-N-nitrosoguanidine | 70-25-7| –      | CHL        | +           | +             | Several| +           | (11) (17)       |

*Concurrent test indicates that the test was performed concurrently with a control treatment. NTP database refers to the National Toxicology Program database.
| Class | Chemical                        | CAS No. | S9 mix | Cells, MNT | MNT | Overall S9 | Cells, CAT | CAT | Overall CAT | References, MNT | References, CAT |
|-------|--------------------------------|---------|--------|------------|-----|------------|------------|-----|-------------|----------------|-----------------|
| CL    | 1-Methyl-1-nitrosourea         | 684-93-5| –      | Several    | +   | –          | Several    | +   | –           | (10)           | (10)            |
| NG    | Methylurea                     | 598-50-5| –      | V79        | –   | –          | CHO        | +   | –           | (10)           | (10)            |
| CL    | Mitomycin C                    | 50-07-7 | –      | V79        | +   | –          | Several    | +   | –           | (17)           | (17)            |
| CL    | Monocrotaline                  | 315-22-0| –      | Rat hepatocytes | +   | +          | CHO        | +   | +           | (17)           | (17)            |
| NG/C  | Nafenopin                      | 3771-19-5| –      | Rat hepatocytes | –   | –          | Rat fibroblasts | –   | –           | (17)           | (17)            |
| NG/C  | Nalidixic acid                 | 389-08-2| –      | CHO        | –   | –          | CHO        | –   | –           | (53) NTP database | (9)             |
| CL    | b-Naphthoquinoline             | 85-02-9 | –      | CHO        | +   | –          | CHO        | +   | +           | (17)           | (17)            |
| CL/NC | N-(1-naphthyl)ethylenediamine. |        | –      | CHO        | –   | –          | CHO        | +   | +           | (22) NTP database |                |
| CL    | Neocarcinostatin               | 9014-02-2| –      | Several    | +   | +          | Several    | +   | +           | (10)           | (10)            |
| CL    | Nickel acetate                 | 373-02-4| –      | CHL        | +   | –          | FM3A       | +   | +           | (17)           | (17)            |
| CL    | Nickel chloride                | 7718-54-9| –      | CHL        | +   | –          | FM3A       | +   | +           | (17)           | (17)            |
| A     | Nitrotriacetic acid            | 139-13-9| –      | Cl-1       | +   | +          | CHO        | –   | –           | (54)           | (42)            |
| E     | o-Nitroaniline                 | 88-74-4 | –      | CHO        | –   | –          | CHO        | –   | –           | i (11)          | (55)            |
| CL/NC | 4-Nitroantranilic acid         | 619-17-0| –      | CHO        | –   | –          | CHO        | +   | +           | (22) NTP database |                |
| CL    | 2-Nitrofluorene                | 607-57-8| –      | CHL        | Weak + | +          | CHL        | +   | –           | (11)           | (56)            |
| CL/NC | 4-Nitro-o-phenylenediamine     | 99-56-9 | –      | CHL        | +   | +          | CHL        | +   | –           | (10)           | (10)            |
| G’/NC | 3-Nitropropionic acid          | 504-81-1| –      | CHO        | –   | –          | CHL, ChoE | +   | +           | (22) NTP database | (22)            |
| CL    | 4-nitroquinoline-N’-oxide      | 56-57-5 | –      | L5178Y     | +   | +          | Several    | +   | +           | (20)           | (17)            |
| CL    | N-nitrosodiamethylenediamine   | 55-18-5 | –      | Rat hepatocytes | +   | +          | CHL, CHL | +   | +           | (31)           | (17)            |
| NG/C  | N-(1-deethyl)nitrosamine       | 86-30-6 | –      | CHO        | –   | –          | CHO        | –   | –           | (58)           | (44)            |
| CL    | m-Nitroso-2-fluorene           | 99-08-1 | –      | CHL        | Weak + | +          | CHL        | +   | –           | (11)           | (17)            |
| CL    | o-Nitrotoluene                 | 88-72-2 | –      | CHL        | +   | +          | CHL        | +   | –           | (11)           | (17)            |
| CL    | p-Nitrotoluene                 | 99-99-0 | –      | CHL        | –   | –          | CHL        | +   | –           | (11)           | (17)            |
| CL    | Norfloxacin                    | 85344-55-4| –      | 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        | +   | +           | (17)           | (17)            |
| G’    | Phenobarbital                  | 50-06-6 | –      | Rat hepatocytes | –   | –          | CHO, CHL-L | +   | +           | (31)           | (17)            |
| CL/NC | Phenol                         | 108-95-2| +      | CHL        | Weak + | +          | CHO        | +   | +           | (11)           | (60)            |
| CL    | Phenolphthalein                | 77-09-8 | –      | MCL-5      | +   | +          | CHO        | +   | +           | (62) NTP database |                |
| CL    | m-Phenylenediamine             | 108-45-2| –      | CHL        | +   | +          | CHL        | +   | –           | Concurrent test* | (17)            |
| CL    | p-Phenylenediamine             | 106-50-3| +      | CHL        | +   | +          | CHL        | +   | –           | (17)           | (17)            |
| CL/NC | p-Phenylenediamine.2HCl         | 624-18-0| +      | CHO        | +   | +          | CHO        | –   | –           | (22) NTP database |                |
| CL    | Potassium bromate              | 7758-01-2| –      | CHL        | +   | +          | CHL        | +   | –           | Concurrent test* | (17)            |
| NG/NC | Promethazine.HCl               | 58-33-3 | +      | V79        | E   | E          | CHO        | –   | –           | (63)           | (36)            |
| NG/NC | Pyrene                         | 129-00-0| –      | V79        | –   | –          | Several    | –   | –           | (7)            | (17)            |
| Class | Chemical                          | CAS No. | S9 mix | Cells, MNT | MNT | Overall MNT | S9 Cells, CAT | CAT Overall | References, MNT | References, CAT |
|-------|----------------------------------|---------|--------|------------|-----|-------------|---------------|-------------|-----------------|-----------------|
| A/CL  | Pyrimethamine                    | 58-14-0 | –      | SHE, HepG2 | –   | –           | CHL, CHO, DON | –           | (10)            | (10)            |
|       |                                  |         | +      | CHO        | –   | –           | CHO, CHL      | –           | (18)            | (10)            |
|       |                                  |         | +      | L5178Y     | –   | –           | CHL           | –           | (10)            | (10)            |
| A     |                                  |         | +/-    | HULY       | –   | –           | CHL           | –           | (10)            | (10)            |
|       | Retinol acetate                  | 127-47-9| –      | V79        | –   | +           | HULY          | +           | (25)            | (64)            |
| A/NC  | Rotenone                         | 83-79-4 | – +/-  | V79        | –   | +           | CHO           | TC          | (7)             | (17)            |
|       |                                  |         |        | V79        | –   | –           | CHO           | +/-         |                 | (5)             |
| NG/C  | Safrole                          | 94-59-7 | –      | +         | +   | +           | +             | –           | (18)            | (17)            |
|       | Sodium chloride                  | 7647-14-5| –     | CHL        | –   | –           | –             | –           | –               | –               |
|       |                                  |         | +      | 16 MA      | –   | –           | 16 MA         | –           | (11)            | (17)            |
| NG/C  | Tetrachloroethylene              | 127-18-4| –      | CHL        | –   | –           | –             | –           | (11)            | (17)            |
|       |                                  |         | +      | 16 MA      | –   | –           | 16 MA         | –           | (68)            | (68)            |
| NG/C  | 12-0-Tetradecanoylphorbul-13-acetate | 16561-29-8 | –    | CHL        | –   | –           | –             | –           | (11)            | (17)            |
| A     | Thiabendazole                    | 148-79-8| –      | V79        | –   | +           | CHO, CHL      | TC          | (7)             | (17)            |
|       |                                  |         | +      | V79        | –   | +           | CHO           | +           |                 | (17)            |
| NG/C  | Titanium dioxide                 | 13463-67-7| –   | CHO        | –   | –           | –             | –           | (23)            | (60)            |
|       |                                  |         | –      | V79        | –   | –           | –             | –           | (69)            | (17)            |
| CL    | Triamterene                      | 396-01-0| –      | Don-6      | –   | +           | –             | +           | (17)            |                 |
|       | 1,1,2-Trichloroethane            | 79-00-5 | –      | H2E1, MCL-5| +   | –           | –             | +           | (25)            |                |
| A     | 1,1,2-Trichloroethylene (with and without epichlorhydrin) | 79-01-6 | –      | MCL-5      | +   | i           | –             | –           | (25)            | (24)            |
| CL    | 1,2,3-Trichloropropane            | 96-18-4 | –      | MCL-5      | +   | i           | –             | +           | (7)             | (25)            |
|       |                                  |         |        | V79        | –   | –           | –             | (25)        | (25)            |                 |
| NG/NC | Triphenyltin hydroxide           | 76-87-9 | –      | CHO        | +   | –           | –             | –           | (22)            |                 |
|       | Urethane                         | 51-79-6 | –      | CHO        | +   | –           | –             | –           | (11)            | (5)             |
| A     | Vinblastine                      | 143-67-9| –      | CHL        | +   | –           | Don           | +           | (11)            | (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.

aChromosomal aberration tests were performed concomitantly under the same experimental condition.

bLack of clear genotoxicity may be due to unusual in vivo metabolism in tumour target tissue.

cThe overall call takes into consideration the numerical aberration data.

dPositive results obtained only at the extremely high concentrations (≥10 mM) were excluded.

eMore clearly genotoxic in systems other than those detecting A or CL.
phenyldiamine and phenacetin.

The results obtained for the six compounds classified as pure aneugens (A) are shown in Table V. All six compounds were negative in the ESAC and positive in the MNT CAT. This conclusion was unanimously endorsed by all members of the ESAC.

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

| MNT | CAT | + | - | Total |
|-----|-----|---|---|------|
| +   | 61  | 0 | 61|
| -   | 9   | 1 | 10|
| Total | 70 | 1 | 71|

*p-Chloroaniline (not convincingly clastogenic), 2-chloro-4-nitroaniline, coumarin, dichloroacetic acid, 2-methyl-4-nitroaniline, N-(1-naphthyl)ethylenediamine2HCl, 4-nitroantranilic acid, 4-nitro-o-phenylenediamine and phenacetin.

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-nitroantranilic acid, 4-nitro-o-phenylenediamine and phenacetin were negative in the in vitro CAT but positive in the in vitro MNT.

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

| MNT | CAT | + | - | Total |
|-----|-----|---|---|------|
| +   | 21  | 6  | 27|
| -   | 0   | 0  | 0 |
| Total | 21 | 6 | 27|

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

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

| MNT | CAT | + | - | Total |
|-----|-----|---|---|------|
| +   | 1   | 3  | 4 |
| -   | 1   | 10 | 11|
| Total | 2 | 13 | 15|

Safrole.

ASAcarbinol, n-butyl-N-(3-carboxypropyl)nitrosamine and triphenyltin hydride.

Nafenopin.

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 | √ |
| Within-laboratory reproducibility | √ |
| Transferability | √ |
| Between-laboratory reproducibility | √ |
| Predictive capacity (concordance) | √ |
| Applicability domain | √ |

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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