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REPORT OF THE JACVAM INITIATIVE INTERNATIONAL VALIDATION STUDIES OF THE IN VIVO RODENT ALKALINE COMET ASSAY FOR THE DETECTION OF GENOTOXIC CARCINOGENS

Series on Testing and Assessment

No. 196

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REPORT OF THE JACVAM INITIATIVE INTERNATIONAL VALIDATION STUDIES OF THE *IN VIVO* RODENT ALKALINE COMET ASSAY FOR THE DETECTION OF GENOTOXIC CARCINOGENS

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Environment Directorate
ORGANISATION FOR ECONOMIC CO-OPERATION AND DEVELOPMENT
Paris 2014

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FOREWORD

This document presents the validation report of the *In vivo* Mammalian Alkaline Comet Assay. It describes the main validation study (4th phase) while the 1st to 3rd phases (pre-validation steps) of the validation study, are reported separately in document No. 195 in the Series on Testing and Assessment. The present document includes three parts, the 1st step of phase 4, the 2nd step of phase 4, as well as an addendum that has been developed in response to the peer review report (document No. 197 in the Series on Testing and Assessment).

The project for developing a Test Guideline for the *in vivo* Mammalian Alkaline Comet Assay was proposed by Japan and included in the work plan of the Test Guidelines Programme in 2008. The pre-validation report and the validation report were submitted for peer review to a subgroup of the expert group on the comet assay in January 2013. This validation report and the peer review report were endorsed by the Working Group of the National Coordinators of the Test Guidelines Programme (WNT) at its 25th meeting in April 2013.

During the course of 2013, and in parallel with the development of the Test Guideline, the expert group on the comet assay addressed the peer review comments based on further analyses or literature review, and an addendum to the validation report was developed which compiles the responses from the expert group. The addendum was submitted separately to the WNT and endorsed at its 26th meeting in April 2014. The Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology agreed to the declassification of the validation report, including the addendum on 7th July, 2014.

This document is published under the responsibility of the Joint Meeting of the Chemicals Committee and the Working Party on Chemicals, Pesticides and Biotechnology.

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Title: Report of the JaCVAM initiative international validation study of the *in vivo* rodent alkaline Comet assay for the detection of genotoxic carcinogens: the 4th (definitive) phase-1st step

Issued: Yoshifumi Uno, D.V.M., Ph.D., and a Validation Management Team (VMT) member

Notes: this document is prepared to summarize the *in vivo* Comet assay validation process and results in the 4th (definitive) phase-1st step. The methods are briefly mentioned in this document, because the details are described in the study protocol and the study plan (Appendices 1 and 2).

1. Introduction

1. The *in vivo* rodent alkaline Comet assay is used worldwide for detecting DNA damage as evidenced by strand breaks. The assay can be applied to the investigation of genotoxic potential of test chemicals, and is currently identified as a second *in vivo* genotoxicity assay in the ICH-S2(R1) guidance (2012) along with the more usual *in vivo* micronucleus test in bone marrow or peripheral blood. The Comet assay protocol has been discussed in the meetings of the International Workshops on Genotoxicity Testing (IWGT) and the International Comet Assay Workshop (ICAW), and consensus articles have been published (Tice, et al., 2000, Hartmann, et al., 2003, Burlinson, et al., 2007).

2. The assay, however, has not been validated formally with a standardized study protocol. In addition, since reports on the predictive capability of the *in vivo* rodent Comet assay for carcinogenicity are limited (Sasaki, et al., 2000, Sekihashi, et al., 2002, Kirkland, et al., 2008), the investigation of predictive capability in multiple laboratories using one validated study protocol would be more useful to understand the overall performance of the assay. The Japanese Environmental Mutagen Society/the Mammalian Mutagenicity Study Group (JEMS/MMS) decided to organise an (international) collaborative study of the *in vivo* Comet assay in 2003, and conducted a preliminary collaborative study on the Comet assay procedure, notably a comparison of assay results between whole cells and isolated nuclei (Nakajima, et al., 2012). At the same time other groups of scientists expressed a wish to establish an OECD guideline for the Comet assay. A coordinated validation effort for the *in vivo* Comet assay was therefore required, and so the Japanese Center for the Validation of Alternative Methods (JaCVAM) organized an international validation study commencing in April, 2006. This was done in cooperation with the U.S. National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), the European Centre for the Validation of Alternative Methods (ECVAM), and JEMS/MMS.

3. The purpose of the validation study was to evaluate the ability of the *in vivo* Comet assay to identify genotoxic chemicals as a potential predictor of rodent carcinogenicity, to demonstrate acceptable intra- and inter-laboratory reproducibility, and to confirm its applicability domain. At the same time it was hoped to consider the value of the *in vivo* Comet assay as an alternative follow-up assay to more the commonly used *in vivo* rodent Unscheduled DNA Synthesis (UDS) assay. The ultimate goal of this validation effort was to establish an OECD guideline for the *in vivo* rodent alkaline Comet assay.

2. Background and Purpose

4. In the 3rd phase of the *in vivo* Comet assay validation study, three coded test chemicals and EMS, the positive control, were assayed in four leading laboratories in accordance with the Comet assay protocol-version 13, and good intra- and inter-laboratory reproducibility was demonstrated. However, it was considered that reproducibility could be further improved, and there was also a wish to combine the detection of DNA damage using the Comet assay and detection of micronuclei in peripheral blood, using the standard micronucleus assay, in the same animals. This could significantly reduce the use of animals for *in vivo* genotoxicity testing. The protocol was further optimized and revised to version 14 (Appendix 1) which involved 3 treatments (0, 24, and 45 h) with a test compound (except for the positive control – see Section 6-2) before collecting liver and glandular stomach tissue samples at 48 h after the first treatment.

5. In the 1st step of the 4th phase validation study (Phase 4-1), the purpose was to examine the extent of reproducibility and variability of assay results among laboratories using coded test chemicals and the positive control EMS, when experiments are conducted in accordance with the Comet assay protocol-version 14 (see the study plan: Appendix 2).

3. Experimental Period

May-December, 2009

4. Participant Laboratories

6. Thirteen laboratories* participated in the 1st step of 4th phase validation study, which included four leading laboratories[#] that were very experienced in the Comet assay plus nine laboratories that were approved following the recruitment process (described in the pre-validation report) for this phase of the validation study.

* Merck Research Laboratories[#] (code: Lab B), BioReliance[#] (Lab C), Huntingdon Life Sciences[#] (Lab D), Food and Drug Safety Center[#] (Lab E), The Institute of Environmental Toxicology (Lab F), Novartis Pharma (Lab G), AstraZeneca (Lab H), Sumitomo Chemical (Lab I), Mitsubishi Chemical Medience (Lab J), Janssen R&D (Lab K), Health Canada (Lab L), Covance (Lab M), and Bayer Schering Pharma (Lab N)

5. Success Criteria in the Study Plan (Appendix 2)

5-1. To obtain positive results in all positive control groups in all testing facilities.

5-2. To obtain consistent positive or negative results in testing facilities that examined the same test chemical.

6. Materials and Methods

7. Outlines of the materials and the methods are described in this section, and the details are referred to in the validation study protocol version 14 (Appendix 1) and the study reports prepared by each laboratory.

8. A study protocol for the Comet assay was prepared in each laboratory in accordance with the validation study protocol v.14. The experiments proceeded in each facility based on their own study protocol and SOPs.

6-1. Animal species, strain, and sex

9. Rats were selected in this validation effort because they are the most popular species for toxicology studies. Crl:CD(SD) rats were used, and since no significant gender differences in response were expected, only male rats were used.

6-2. Test chemical, vehicle, dose level, and administration

10. Ethyl methanesulfonate (EMS) was used as the positive control because it is a well-known genotoxic chemical, active in multiple organs, and had been used in the earlier pre-validation phases. EMS was dissolved in physiological saline, and administered to rats at a dose level of 200 mg/kg twice (21 hr interval) orally by gavage. It was administered twice rather than 3 times (as for the coded test chemicals) in order to obtain data for EMS under the same administration scheme as had been used in the pre-validation phases.

11. The VMT considered that, in this phase 4-1, examination of the same chemicals used in the 3rd phase pre-validation study (Phase 3) would provide useful information about the reproducibility of results between different phases of validation studies as well as between laboratories. Therefore, of the four coded test chemicals that were used in this study, three were the same chemicals used in Phase 3, i.e. EMS (coded as well as being the positive control), *N*-methyl-*N*-nitrosourea (MNU), and D-mannitol (MA). The fourth chemical was 2-acetylaminofluorene (2-AAF) which was selected as a typical mutagen that requires metabolic activation and produces bulky adducts, i.e. a different mode of action from the alkylating agents, EMS and MNU. The vehicle for each coded test chemical was selected by each testing facility based on preliminary solubility assessments. The dose levels were also decided by each facility based on the results of a preliminary dose range finding study, although the VMT provided some toxicological information from the published literature such as LD_{50} values if

available, to assist the selection of preliminary dose levels. Each coded test chemical was administered to rats at three dose levels, at three time points (0, 24 and 45 hours, i.e. 24 and 21 hours intervals) by oral gavage. This administration regimen was designed to allow combination of the micronucleus and Comet endpoints in the same animals in consideration of the 3R's principle for animal use. For these studies the investigation into micronucleus induction was optional and micronucleus data are not included in this validation study report. However, if examined, the micronucleus data may be included in the study reports prepared by the testing facilities; Appendix 3.

12. Each coded test chemical was examined in three or four laboratories to evaluate the extent of reproducibility and variability of the assay results between laboratories using the coded test chemicals and the positive control EMS. The vehicle and the dose levels of the test chemicals are summarized in Table 1.

Table 1 Test chemical, laboratory tested, vehicle, and dose levels

Test chemical	Lab. Code	Vehicle	Dose level (mg/kg/day)
EMS	Lab C	Water for injection	75, 150, 300
	Lab J	Physiological saline	62.5, 125, 250
	Lab M	Physiological saline	100, 200, 400
	Lab N	Corn oil	75, 150, 300
MNU	Lab E	Physiological saline	25, 50, 100
	Lab F	Physiological saline	30, 60, 120
	Lab I	Physiological saline	50, 100, 200 [1)] Additional: 6.25, 12.5, 25
MA	Lab D	Purified water	500, 1000, 2000
	Lab G	Water	500, 1000, 2000
	Lab H	Physiological saline	500, 1000, 2000
	Lab L	Physiological saline	400, 800, 1600
2-AAF	Lab B	0.5% CMC aqua solution	250, 500, 1000 [2)]
	Lab H	Corn oil	75, 150, 300 [3)]
	Lab K	Corn oil	125, 250, 500

1) Animal death at 200 mg/kg/day. Cytotoxicity for the stomach was noted in all dose levels, and the additional study was done at lower dose levels, namely 6.25, 12.5, and 25 mg/kg/day. There was no cytotoxicity at 6.25 mg/kg.

2) Lab B selected the highest dose in consideration of the amount of test chemical delivered. They suggested that a higher dose level could have been evaluated because no animal toxicity was found at 1000 mg/kg/day.

3) Lab H knew the identity of the chemical before the assay was commenced due to the information from customs (when

JaCVAM sent test chemicals to overseas facilities, the customs checked it and then informed the facility of the chemical name), and selected the dose levels based on published toxicity information of 2-AAF. However, no toxicity was found in animals up to the highest dose level.

6-3. Organs analyzed

13. Liver and stomach (glandular stomach) were selected for this validation effort because the former is the primary organ for the metabolism of absorbed chemicals, and the latter is a site of first contact of chemicals after oral administration. These organs were recommended for screening for genotoxic chemicals in the previous discussion in ICAW (Hartmann, et al., 2003).

6-4. Data-acceptance criteria

6-4-1. Negative control

14. Means of %DNA in tail should be 1-8% in the liver and 1-30% (preferably 1-20%) in the stomach.

6-4-2. Positive control, EMS, 200 mg/kg, twice p.o.

15. Effect (difference of means of % tail DNA between groups of EMS and vehicle control) is statistically significantly increased and is at least 5% higher than negative control levels in the liver and the stomach (primary criteria); and Effect (ratio of means of % DNA in tail between groups of EMS and vehicle control) is 2-fold or higher in the liver and the stomach.

6-5. Data analysis

16. % DNA in tail was used as the primary endpoint of this validation study, because it is considered linearly related to the DNA break frequency over a wide range of damaged DNA levels (Hartmann, et al., 2003).

17. Three conceptual key terms, “Endpoint”, “Estimate” and “Effect” were defined and used in the data analysis. Briefly, Endpoint is defined as individual observed values for a parameter such as % DNA in tail. Estimate is defined as a mean calculated with values of Endpoint in each animal. Effect is defined as difference (hereafter designated as Effect (diff.)) or ratio (hereafter designated as Effect (ratio)) of a mean of Estimates between a negative control group and a treatment group. A general purpose of data analysis in validation studies is to investigate how large is the variation that exists among data from several testing facilities, and Effect is considered as a good indicator to understand the variability of Comet assay parameters among testing facilities. VMT noticed through Phases 1 to 3 of the pre-validation studies that Effect (diff.) was more meaningful for the comparison of variability than Effect (ratio), because Effect (ratio) depended on the magnitude of the negative control values (i.e.

lower negative control values produce higher Effect (ratio) more easily) and could lead to a misleading evaluation of responses induced with a test chemical. Therefore Effect (diff.) was considered the main response criterion with which to evaluate the assay results.

18. Because many of the participating laboratories did not have extensive historical negative control data with the exact protocol being used, statistical analysis was considered to be the most appropriate way to determine a positive response in this validation trial. Dunnett's test (two-sided, $P<0.05$) and linear Trend test (two-sided, $P<0.05$) were applied to Effect (diff.) in the groups of coded test chemicals. The two-sided analysis was used because both increases and decreases in the comet parameter could be detected. A decreased % DNA in tail was expected to be a good index to detect cross-linking agents. For the positive control group, Student's t-test (one-sided, $P<0.025$) was applied to the Effect (diff.).

7. Results

19. In this section only outlines of the study results are described. The details are given in the study reports written by each laboratory and the statistical analysis by Dr. Takashi Omori.

7-1. Control groups

20. Figs. 1 and 2 summarize the lab-orderly means of % DNA in tail (Estimate) in the vehicle and positive control EMS groups in the liver and the stomach, respectively.

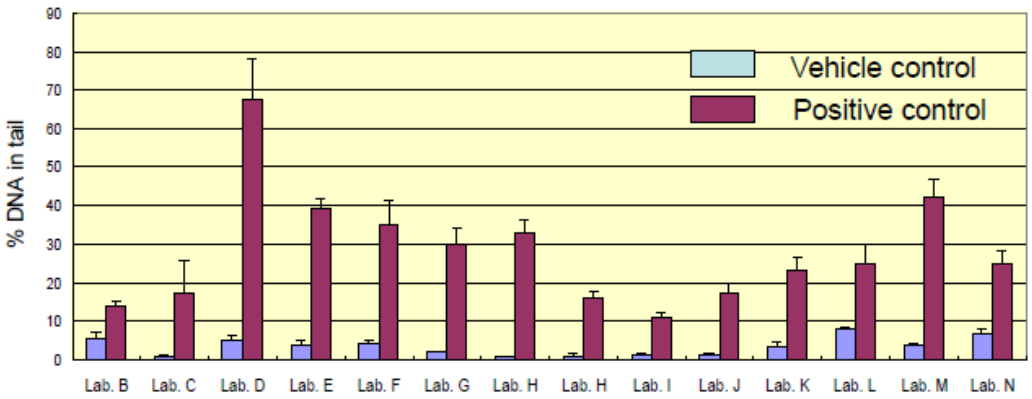


Fig. 1 Estimate (% DNA in tail) in the liver of vehicle control and positive control EMS groups in each lab. Each column shows mean ± S.D. (n=5 animals).

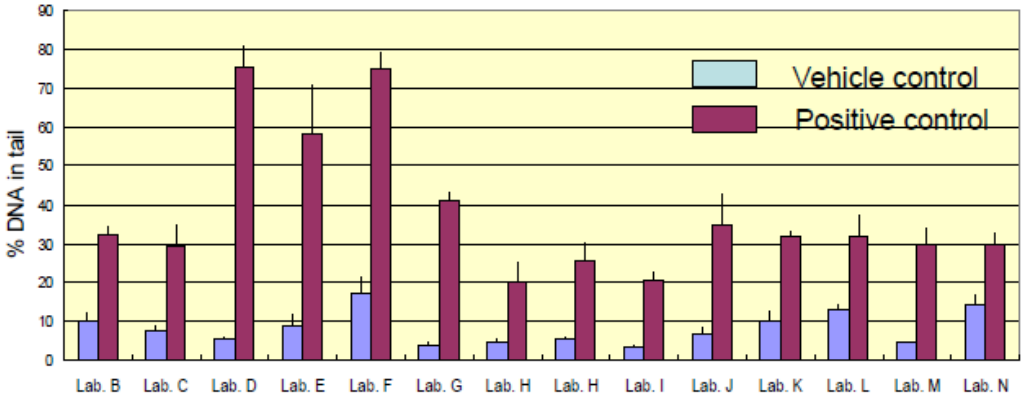


Fig. 2 Estimate (% DNA in tail) in the stomach of vehicle control and positive control EMS groups in each lab. Each column shows mean ± S.D. (n=5 animals).

7-1-1. Vehicle control groups

21. Figs. 3 and 4 are enlarged-figures of the lab-orderly Estimate in the vehicle control groups in the liver and the stomach, respectively. All the values in the stomach satisfied the preferred data acceptance criteria (1-20%), and those in the liver also met the data acceptance criteria (1-8%) except for 2 cases where low values were obtained, namely Lab C (mean value 0.9%) and one of two experiments in Lab H (mean value 0.8%). However, these were considered to be minor deviations from the expected negative control range.

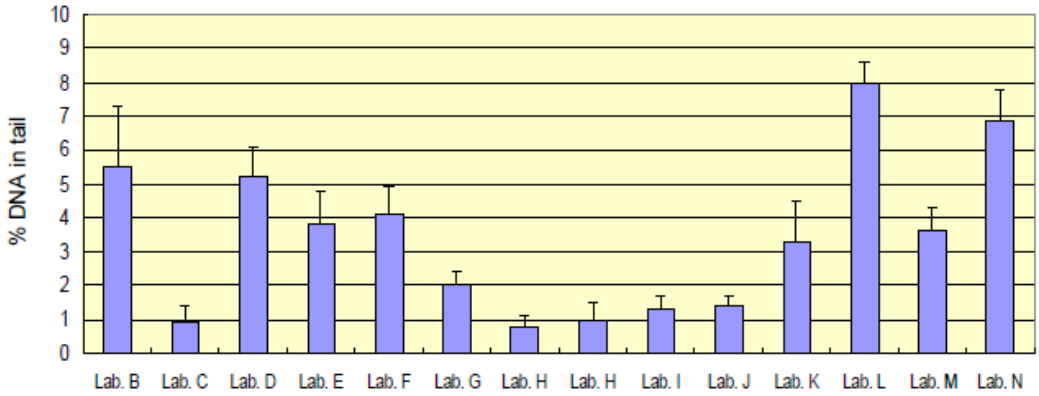


Fig. 3 An enlarged figure of Estimate (% DNA in tail) in the liver of vehicle control group in each lab. Each column shows mean ± S.D. (n=5 animals). All values satisfied the data acceptance criteria 1-8% except Lab. C and one of two experiments in Lab. H.

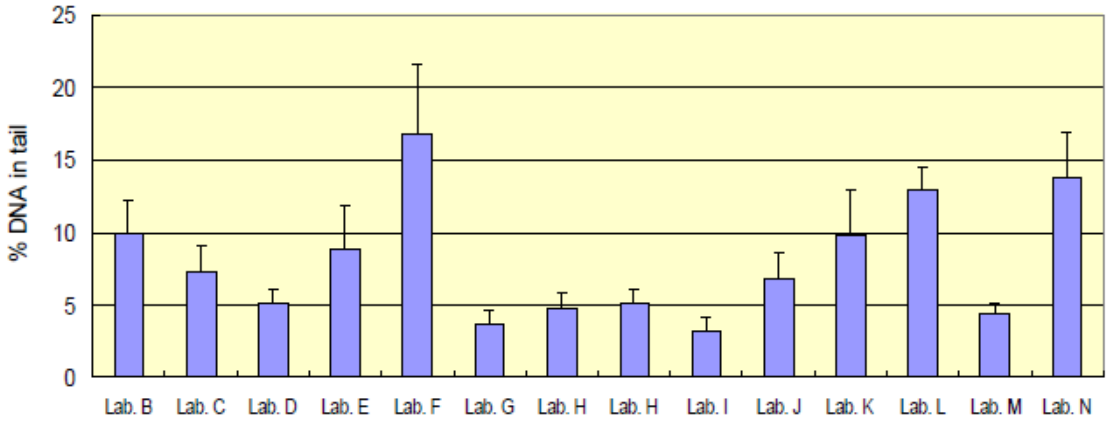


Fig. 4 An enlarged figure of Estimate (% DNA in tail) in the stomach of a vehicle control group in each lab. Each column shows mean ± S.D. (n=5 animals). All values satisfied the preferable data acceptance criteria 1-20%.

7-1-2. Positive control groups

22. Figs. 5 and 6 show Effect (diff.) of mean %DNA in tail between the vehicle control group and the positive control group in the liver and the stomach, respectively. All show statistically significant increases, Student's t-test (one-sided, $p<0.025$) both in the liver and the stomach, and also showed 5% or higher values. Therefore, it was judged that all the positive control values satisfied the primary data acceptance criteria.

23. Figs. 7 and 8 show Effect (ratio) of mean % DNA in tail between the vehicle control group and EMS group in the liver and the stomach, respectively. Since all Effects (ratio) were 2-fold or higher in both organs, it was judged that all the positive control values satisfied the data acceptance criteria.

24. Thus, for this part of the validation exercise it was concluded that participating laboratories could satisfactorily obtain both negative and positive control data that met the data acceptance criteria.

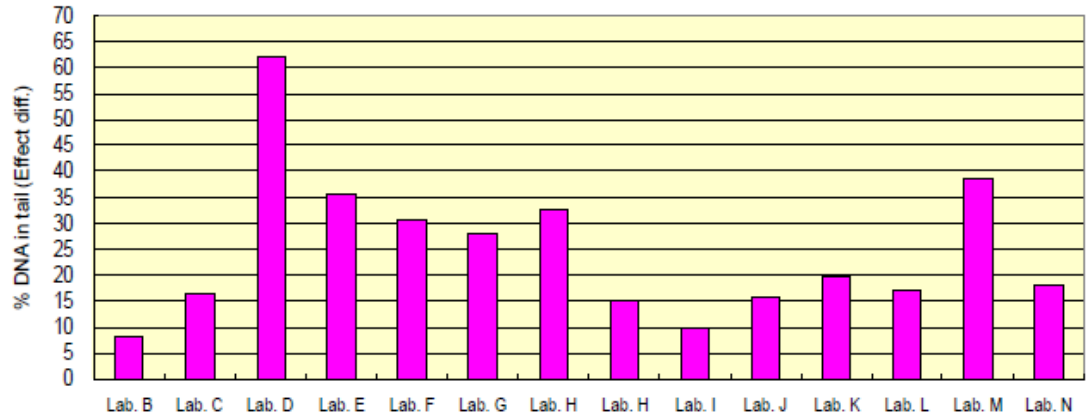


Fig. 5 Effect (difference between means of negative and positive control groups) of % DNA in tail in the liver in each lab. All data show statistically significant increases in Student's t-test (one-sided, $p<0.025$) and 5% or higher values, satisfying the primary data acceptance criteria.

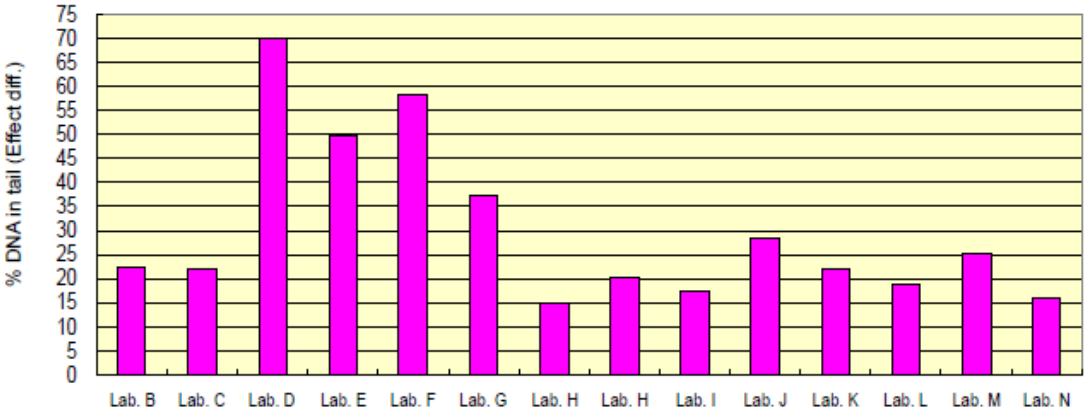


Fig. 6 Effect (difference between means of negative and positive control groups) of % DNA in tail in the stomach in each lab. All data show statistically significant increases in Student's t-test (one-sided, $p<0.025$) and 5% or higher values, satisfying the primary data acceptance criteria.

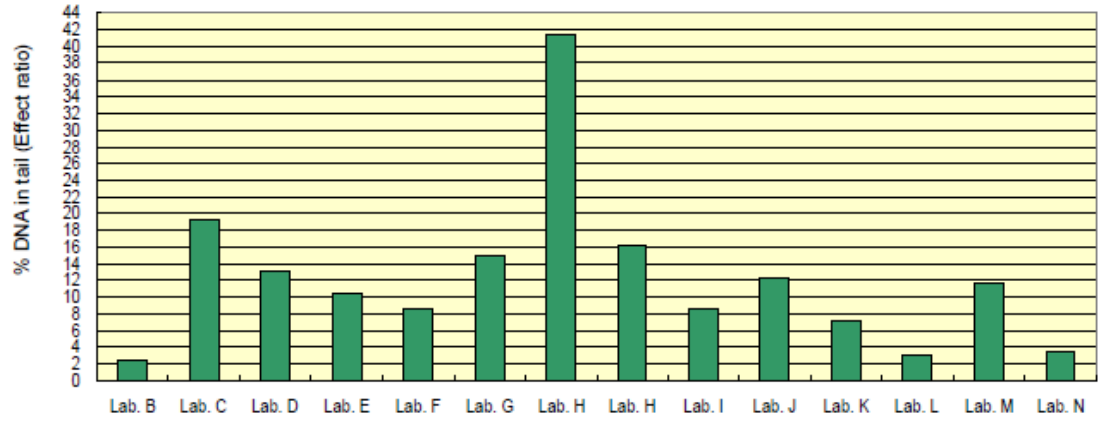


Fig. 7 Effect (ratio between means of negative and positive control groups) of % DNA in tail in the liver in each lab. All data show 2-fold or higher increases, satisfying the data acceptance criteria.

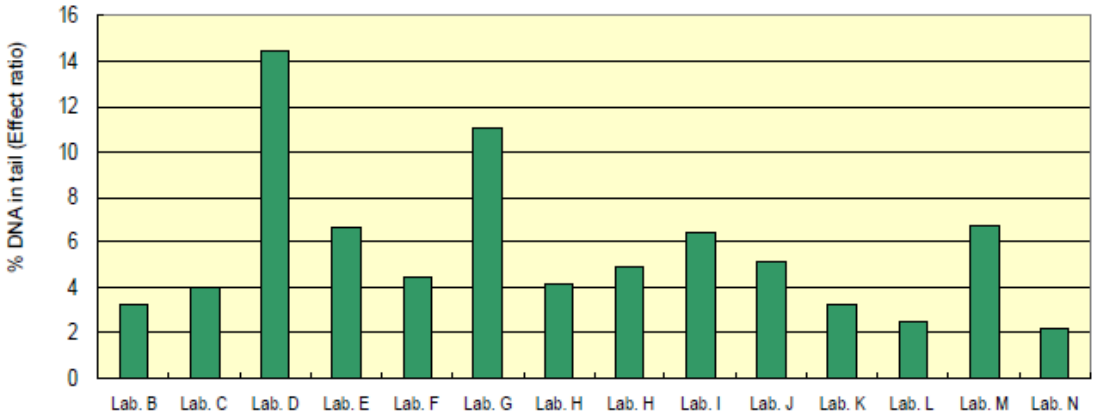


Fig. 8 Effect (ratio between means of negative and positive control groups) of % DNA in tail in the stomach in each lab. All data show 2-fold or higher increases, satisfying the data acceptance criteria.

7-2. Coded test chemical groups

7-2-1. EMS

25. EMS as a coded test chemical was evaluated in four laboratories. Figs. 9 and 10 show the mean % DNA in tail (Estimate) in the vehicle control group and the three dose-level groups in the liver and the stomach, respectively. The vehicle and the dose levels were decided in each laboratory independently based on the preliminary examinations. The same type of aqueous vehicle was selected in two of the four laboratories (note: should be correctly described for Lab M when the reports are available), and similar dose levels were also chosen.

26. All of the Effect (diff.) values were statistically significant and showed dose-dependency in both the liver and the stomach. The magnitude of the responses in both organs was comparable for the same or similar dose levels among four laboratories, except for Lab M where higher values were observed in the liver. The responses were also consistent when compared with the 200 mg/kg dose of EMS used as positive control. Therefore these data demonstrate very good inter-laboratory reproducibility in terms of qualitative response, and generally good reproducibility in terms of quantitative response.

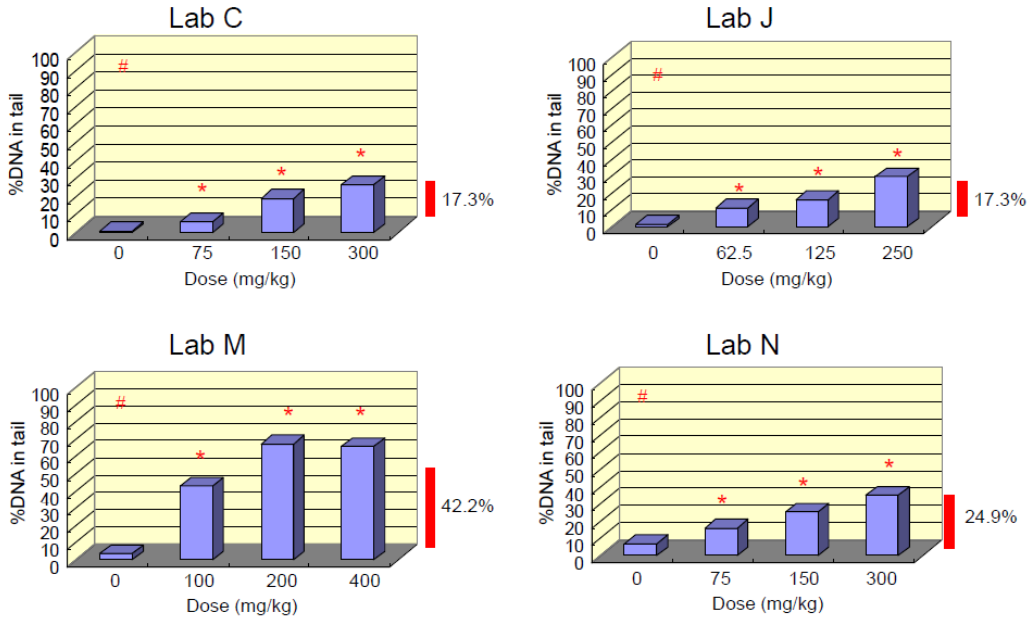


Fig. 9 Estimate (mean of % DNA in tail, n=5 animals) in the liver after EMS administration. Asterisk (*) and sharp (#) indicate statistical significance in Dunnett test (two-sided, $p<0.05$) and linear trend test (two-sided, $p<0.05$), respectively. Red bar shows Estimate (mean of % DNA in tail, n=5) of positive control group.

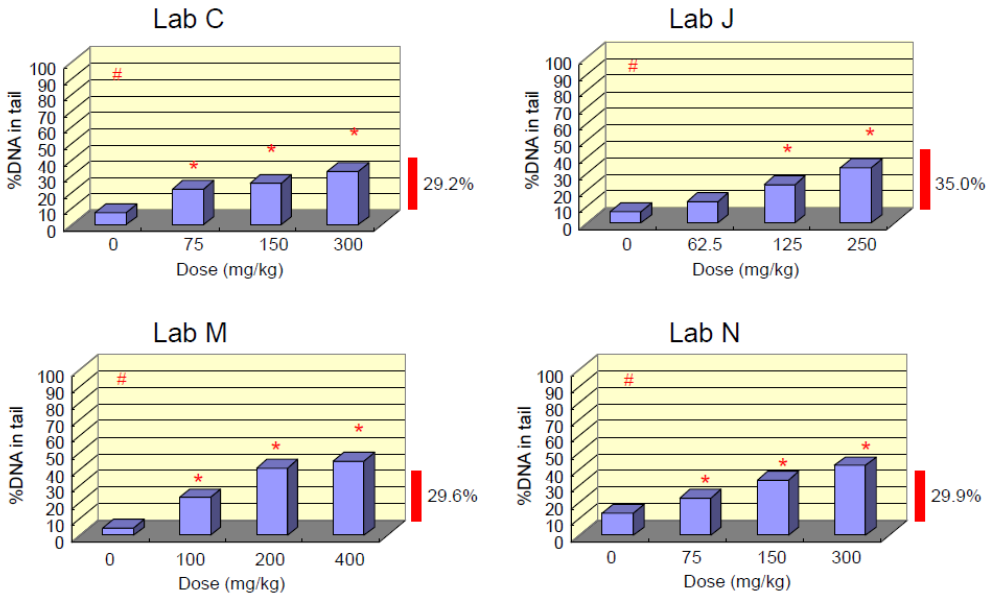


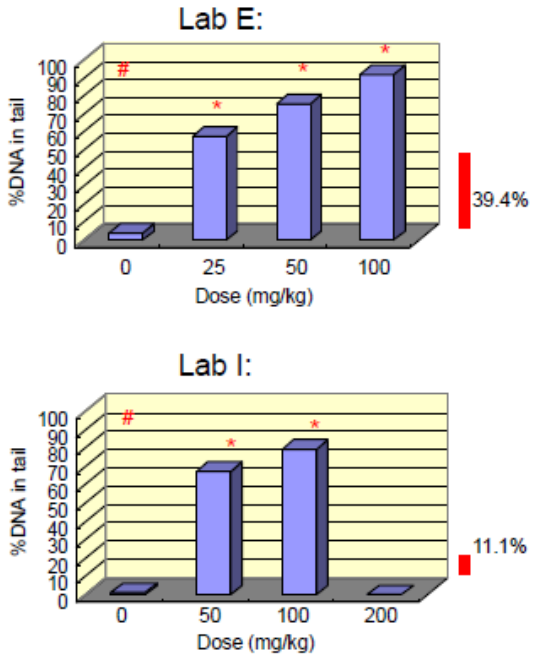
Fig. 10 Estimate (mean of % DNA in tail, n=5 animals) in the stomach after EMS administration. Asterisk (*) and sharp (#) indicate statistical significance in Dunnett test (two-sided, $p<0.05$) and linear trend test (two-sided, $p<0.05$), respectively. Red bar shows Estimate (mean of % DNA in tail, n=5) of positive control group.

27. Following histopathological examination, Lab N reported that for EMS-treated animals hepatocellular hypertrophy was found in the livers of rats in the 300 mg/kg/day group, and inflammatory changes in the stomach at 200 and 300 mg/kg/day in the dose range-finding study. Lab J reported that hepatocellular necrosis (minimal and focal) in the liver was observed in one animal at 62.5 mg/kg/day, and mucosal erosion in the glandular stomach was observed in one animal each at 125 and 250 mg/kg/day. Lab C reported that both tissues showed normal pathology. These observations are considered relevant in terms of the possible impact of cytotoxicity (histopathological changes in target tissues) on the interpretation of Comet assay results.

7-2-2. MNU

28. MNU was evaluated in three laboratories. Figs. 11 and 12 show the mean % DNA in tail (Estimate) in the vehicle control group and the three dose-level groups in the liver and the stomach, respectively. Again the vehicle and dose levels were decided in each laboratory independently. The same vehicle, physiological saline, was used in all laboratories, but different dose levels were selected by each facility for the main studies based on the results of the dose range-finding studies. Although deaths occurred at the highest dose level (350 mg/kg/day) in the range-finding study in Lab I, and therefore Lab I selected 200 mg/kg/day as the highest dose level for the main study, there were no deaths and no clinical signs in animals

at 200 mg/kg/day for 3 days in their dose-finding study, it was considered that the dose selection for the main study was justified.



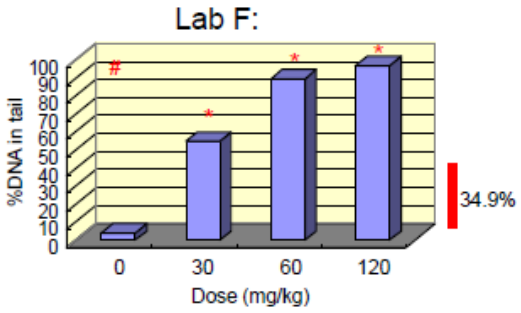
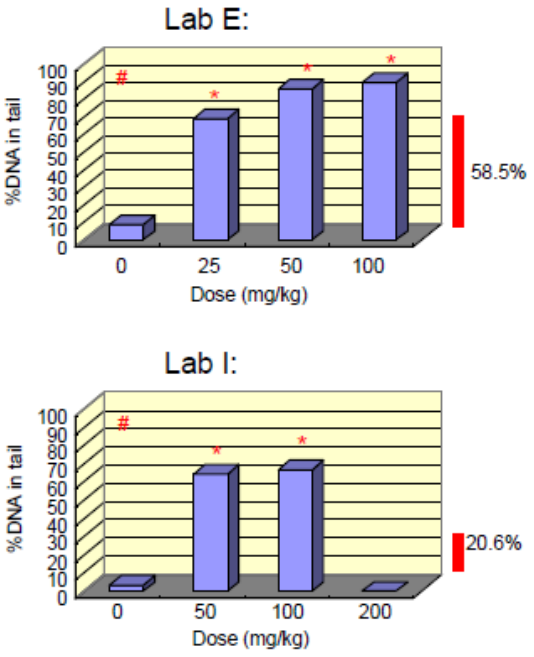


Fig. 11 Estimate (mean of % DNA in tail, n=5 animals) in the liver after MNU administration. Asterisk (*) and sharp (#) indicate statistical significance in Dunnett test (two-sided, $p<0.05$) and linear trend test (two-sided, $p<0.05$), respectively. Red bar shows Estimate (mean of % DNA in tail, n=5) of positive control group.



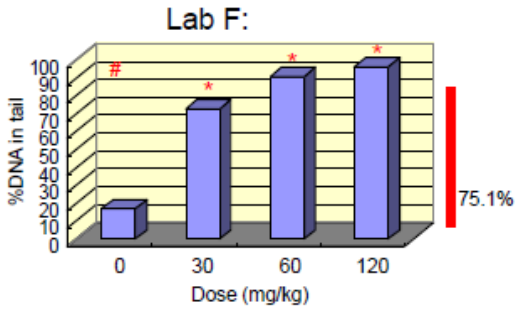


Fig. 12 Estimate (mean of % DNA in tail, n=5 animals) in the stomach after MNU administration. Asterisk (*) and sharp (#) indicate statistical significance in Dunnett test (two-sided, $p<0.05$) and linear trend test (two-sided, $p<0.05$), respectively. Red bar shows Estimate (mean of % DNA in tail, n=5) of positive control group.

29. All of the Effect (diff.) values were statistically significant and showed dose-dependency in both the liver and the stomach. The magnitude of responses in both organs was comparable at the same or similar dose levels across three laboratories. This again demonstrates good qualitative and quantitative reproducibility between laboratories.

30. Regarding cytotoxicity, there were no treatment-related cytotoxic findings in the liver by histopathological examination. In the stomach, cytotoxic changes were observed by histopathology as follows: congestion in lamina propria, degeneration/vacuolar in gastric pits, edema in submucosa and erosion at 50 and 100 mg/kg/day in Lab E; partial depletion of glandular epithelial cells and submucosal edema at all dose levels, and mucosal necrosis at 120 mg/kg/day in Lab F; and congestion/hemorrhage, degeneration/necrosis in mucosal epithelial cell, detachment in mucosal epithelial cell, glandular dilatation and infiltration in inflammatory cell findings at 50 mg/kg/day or above in Lab I. Lab F and Lab I also reported that almost all cells showed “hedgehog” appearance at the high dose levels (and also at the middle dose level in Lab F) in both organs . Again, these observations are considered relevant in terms of the possible impact of cytotoxicity (histopathological changes in target tissues) on the interpretation of Comet assay results.

7-2-3. MA

31. MA was evaluated in four laboratories. Figs. 13 and 14 show the mean % DNA in tail (Estimate) in the vehicle control group and the three dose-level groups in the liver and the stomach, respectively. The vehicle and the dose levels were decided in each laboratory independently. Water or physiological saline was used as the vehicle in all laboratories, and similar dose levels were selected by all facilities based on the result of each dose-finding study.

32. Only Lab L selected a lower top dose (1600 mg/kg/day) because precipitation was noted in the dosing solution above 160 mg/mL (note: Lab L thought that reaching the solubility limit in the vehicle was one of the criteria for selection of the highest dose level, although this was not mentioned in the standard study protocol. VMT did not notice this misunderstanding of Lab L at this stage of the validation study).

33. There were no statistically significant increases or dose-dependency in Effect (diff.) with MA in any of the laboratories, except for Lab D where a statistically significant response was obtained with Dunnett’s test in the stomach at the mid dose 1000 mg/kg/day. However, since there was no dose-response, this was considered not to be a biologically relevant response by both Lab D and the VMT. Thus, the qualitative responses across the 4 laboratories with MA were very reproducible.

34. No histopathology was examined in any laboratories due to the lack of comet responses with this chemical in all testing laboratories.

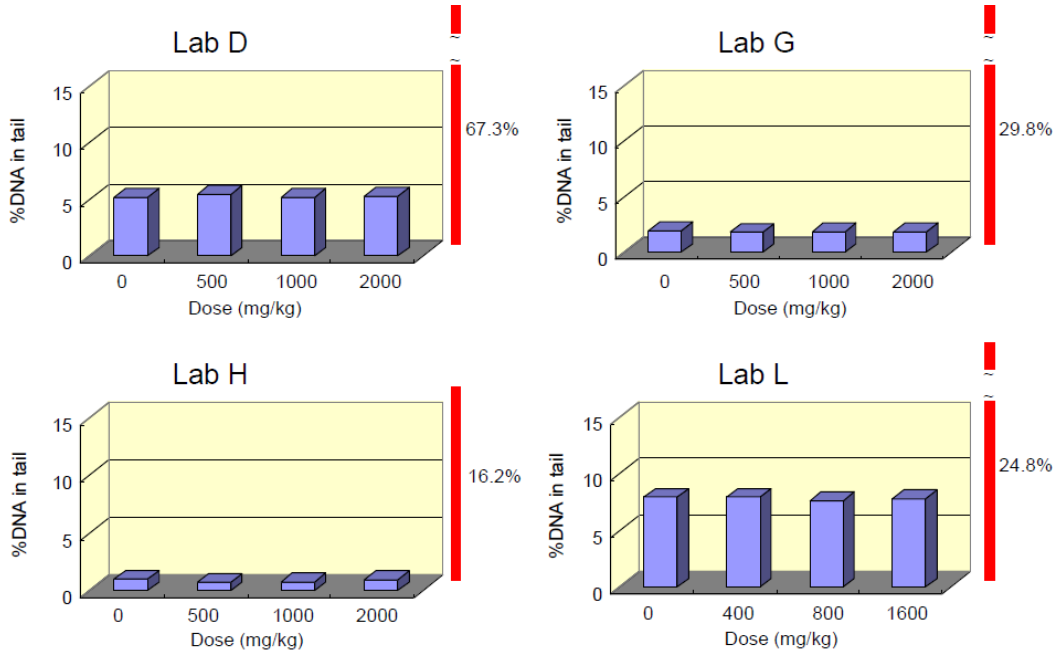


Fig. 13 Estimate (mean of % DNA in tail, n=5 animals) in the liver after MA administration. There was no statistical significance in Dunnett test (two-sided, $p<0.05$) and linear trend test (two-sided, $p<0.05$). Red bar shows Estimate (mean of % DNA in tail, n=5) of positive control group.

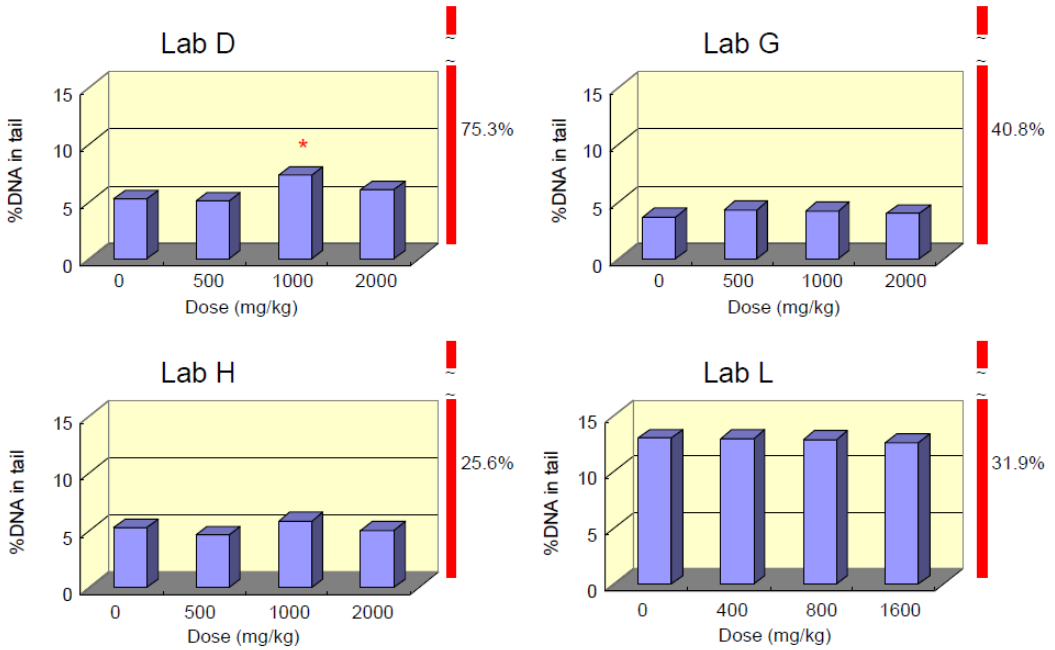


Fig. 14 Estimate (mean of % DNA in tail, n=5 animals) in the stomach after MA administration. Asterisk (*) shows statistical significance in Dunnett test (two-sided, $p<0.05$). There was no statistical significance in linear trend test (two-sided, $p<0.05$). Red bar shows Estimate (mean of % DNA in tail, n=5) of positive control group.

7-2-4. 2-AAF

35. 2-AAF was evaluated in three laboratories. Figs. 15 and 16 show the mean % DNA in tail (Estimate) in the vehicle control group and the three dose-level groups in the liver and the stomach, respectively.

36. The vehicle was selected independently in each laboratory. Lab B selected 0.5% aqueous CMC as the vehicle because this chemical did not dissolve in saline but could be suspended in 0.5% aqueous CMC. Lab H selected corn oil based on the known solubility of 2-AAF, since this laboratory knew the identity of the chemical before the experiment commenced, because the customs office informed the laboratory of the chemical name in their import process. A similar situation occurred at Lab K, which also selected corn oil based on knowledge of the identity of the chemical before the experiment commenced. However, in both cases, although the validation study representative suggested corn oil as a suitable vehicle to the technicians performing the study, they did not inform the technicians of the actual chemical name. Therefore, since the experiments themselves were conducted by technicians under the coded test chemical conditions, the VMT considered that the data on 2-AAF from these 2 laboratories were acceptable for evaluation.

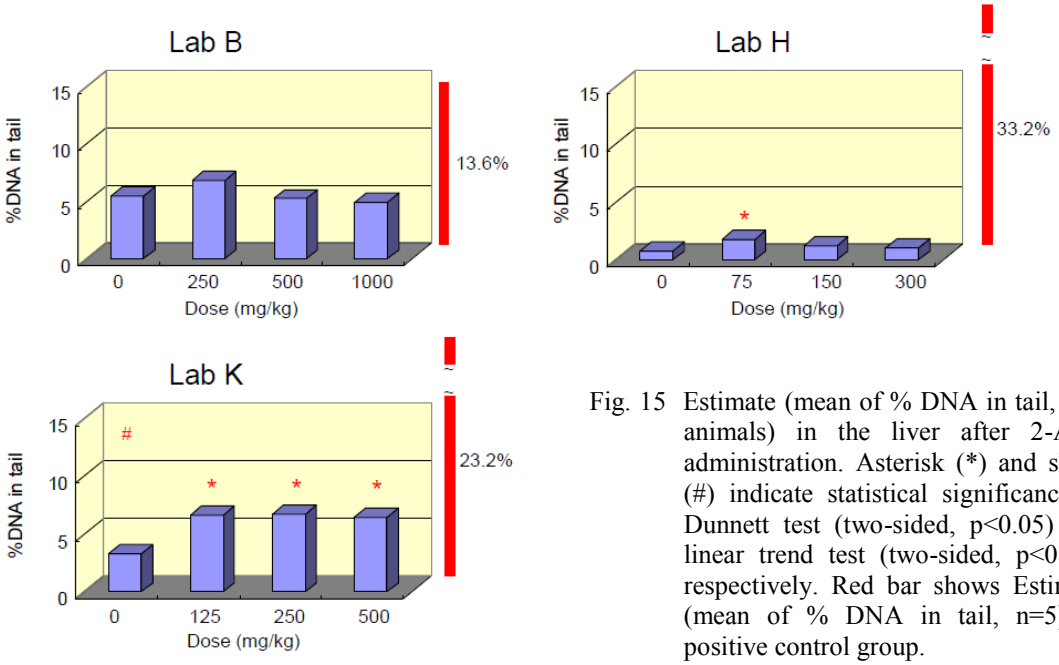


Fig. 15 Estimate (mean of % DNA in tail, n=5 animals) in the liver after 2-AAF administration. Asterisk (*) and sharp (#) indicate statistical significance in Dunnett test (two-sided, $p<0.05$) and linear trend test (two-sided, $p<0.05$), respectively. Red bar shows Estimate (mean of % DNA in tail, n=5) of positive control group.

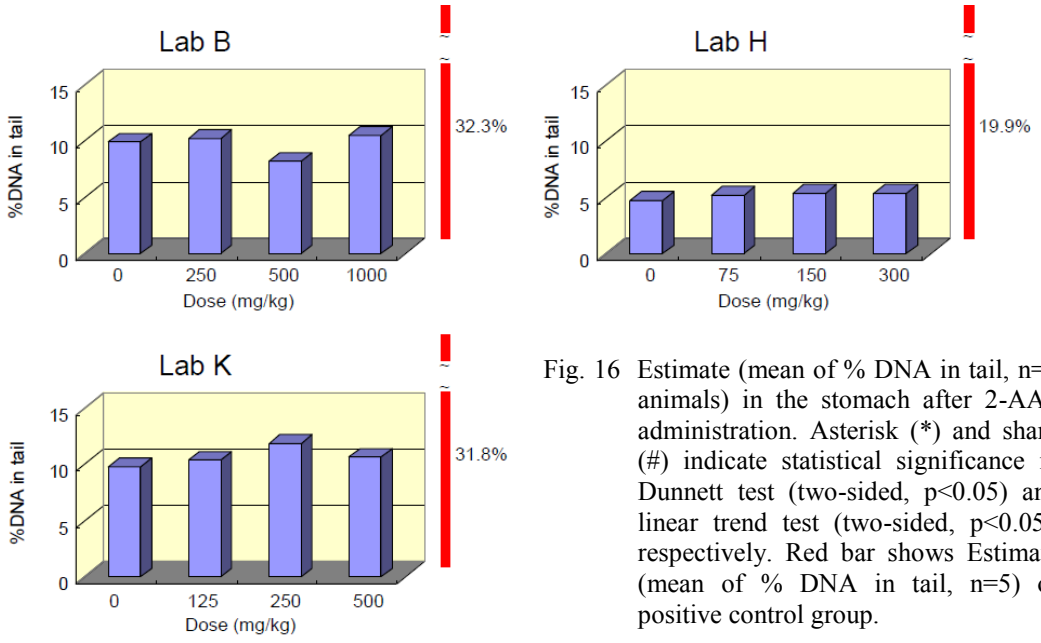


Fig. 16 Estimate (mean of % DNA in tail, n=5 animals) in the stomach after 2-AAF administration. Asterisk (*) and sharp (#) indicate statistical significance in Dunnett test (two-sided, $p<0.05$) and linear trend test (two-sided, $p<0.05$), respectively. Red bar shows Estimate (mean of % DNA in tail, n=5) of positive control group.

37. The dose levels were also determined independently in each laboratory. Dose range-finding studies were only conducted in Labs B and K. In Lab B, no toxic signs were observed up to 1000 mg/kg/day. However, as the amount of test chemical delivered was limited, Lab B performed the Comet assay only up to 1000 mg/kg/day, and not up to 2000 mg/kg/day as had been recommended as an upper limit in the validation study protocol. In Lab K, 500 mg/kg/day was selected as the highest dose level for the dose range-finding study based on the information from the VMT, i.e., mouse oral LD_{50} is 810 or 1020 mg/kg, and suggestions to the technicians by the validation study representative who knew the identity of the chemical. Although a (minor) reduction in body weight and body weight gain was seen at all dose levels, there were no clinical abnormalities up to 500 mg/kg/day. However, Lab K selected 500 mg/kg/day as the highest dose for the Comet assay. Lab H did not do a dose range-finding study because, as explained above, the validation study representative knew the chemical name and selected the dose levels based on the published toxicity information for 2-AAF. In these circumstances, a dose range-finding study would not have been allowed for ethical reasons. The dose selection was therefore justified on this basis, although there was no toxicity seen at the top dose (300 mg/Kg/day) used in the Comet assay.

38. As can be seen in Fig. 16, in the stomach there were no statistically significant increases or signs of dose-dependency in Effect (diff.) for 2-AAF in any of the laboratories. This may be expected for a chemical requiring metabolic activation. In the liver (Fig. 15), Effect (diff.) values were statistically significant with dose-dependency in Lab K, but not in Labs B and H, although a significant increase was seen in the lowest dose group, 75 mg/kg/day, in Lab H. Thus, 2-AAF was not clearly detected as positive in 2 of the 3 laboratories, and therefore the inter-laboratory reproducibility for responses to 2-AAF was poor.

39. In Lab K, histopathology was examined for the liver due to the positive result in the Comet assay. Periportal hepatocellular hypereosinophilia often associated with reduced hepatocellular glycogen was shown to be marginally increased in the highest dose group.

8. Discussion

40. EMS treatment as the positive control induced statistically significant increases in Effect (diff.) in both the liver and the stomach in all thirteen laboratories. In addition, Effect (diff.) and Effect (ratio) were 5% or higher and 2-fold or higher in both organs in all testing facilities, respectively, showing that the positive control completely met the data acceptance criteria. Therefore, the first success criteria, i.e., to obtain positive results in all positive control groups in all testing facilities was satisfied in this validation study.

41. Some discussion would be needed about the slight deviation from the data acceptance criteria on negative control groups for the liver in Lab C (0.9%) and one experiment in Lab H (0.8%). The criteria of 1-8% in the liver were set to detect decreases in % DNA in tail thought to be induced by DNA cross-linker type mutagens. Since Lab C examined EMS, and Lab H examined 2-AAF, and neither of these chemicals are cross-linkers, the VMT considered that the slightly lower values in the negative control groups would not affect the evaluation of these chemicals, and so decided to accept the slightly lower negative control values. Regarding the lower % tail DNA in the liver of the negative control group, Lab I pointed out that it was difficult to keep 1% or higher in the liver because the values became lower experiment by experiment due to the laboratory becoming more technically skilled at cell preparation. The VMT advised Lab I that to keep 1% or higher values would be feasible by prolonged electrophoresis duration and/or higher temperature of alkaline solution, but still below 10°C.

42. Of the four coded test chemicals, EMS, MNU, and MA were evaluated under similar experimental designs in 3 or 4 laboratories and all gave very consistent Comet assay data i.e., EMS and MNU were judged positive, and MA was judged negative. EMS and MNU not only showed good reproducibility of positive responses from a qualitative point of view, there was also generally good quantitative reproducibility. Although MA showed a slight but statistically significant increase in Dunnett's test for the stomach at the mid dose level (1000 mg/kg/day) in Lab D, both Lab D and the VMT judged the response to be biologically insignificant because it showed no dose dependency. The reproducibility of qualitatively negative results with a known non-carcinogen (MA) was therefore also demonstrated. Therefore the second success criteria, i.e. to obtain consistent positive or negative results in different testing facilities examining the same test chemical was satisfied for those three chemicals. In addition, these results were quite consistent with those obtained for the same chemicals in the 3rd phase pre-validation study, confirming the robust reproducibility of the assay both over time and between laboratories.

43. Discussion was needed regarding the results with 2-AAF, which were not as expected and did not show good reproducibility. Negative results in both organs were obtained in Lab B, but Lab B suggested that the higher dose levels up to 2000 mg/kg should be examined because there were no toxic signs in animals up to 1000 mg/kg/day. Lab H examined 2-AAF only up to 300 mg/kg/day based on information in the published literature, and judged it to be negative in both organs. Based on the lack of toxic signs at this dose, and data from the other laboratories, higher doses should have been used. In contrast, statistically significant increases in % DNA

in tail were noted in the liver at all dose levels in Lab K, even though the highest dose level was only 500 mg/kg/day, and a negative result was obtained in the stomach. Since the dose levels used in three laboratories were not similar, the VMT considered that it would be difficult to assess the consistency of assay results in the three laboratories that tested 2-AAF. The judgment of assay results was summarized as three negative calls in the stomach, and one positive call and two negative calls in the liver, based on the statistical analysis. Thus, although there was consistency between the assay results in the stomach, the results were inconsistent in the liver. It would be important to examine higher dose levels of 2-AAF in order to reach more meaningful positive/negative judgments, and to be able to assess inter-laboratory reproducibility with this chemical. Finally, the VMT considered that 2-AAF was judged as inconclusive due to the variable testing conditions. Therefore the VMT decided that 2-AAF would be reexamined in the next step of the 4th phase validation study under the coded test chemical conditions.

44. Questions still remain about how the results of histopathology analysis and % hedgehog should be considered when interpreting Comet assay results. In previous discussions at IWGT, a consensus was obtained, i.e., histopathology is the gold standard to evaluate cytotoxicity in the *in vivo* Comet assay (Hartmann, et al., 2003). However, it is still unclear how to use histopathology for the interpretation of Comet assay results, or even when is the optimum time to examine tissues for histopathological changes. The results with MNU in this phase may provide some important information regarding this interpretation. MNU treatment produced a high frequency of hedgehogs but no histopathological changes in the liver. In contrast, it produced not only increased hedgehogs but also necrosis (identified by histopathology) in the stomach. If hedgehogs are considered to be cells with heavily damaged DNA and/or apoptotic cells, the absence of histopathological changes in the presence of increased % of hedgehogs may indicate that most cells with severe DNA damage continue to be alive, possibly due to protective functions such as DNA repair. In contrast, necrotic findings without increased hedgehogs may indicate that the necrosis is related to cellular toxicity by some functional damage to cell organelles without primary DNA damage. In the case of necrotic findings accompanied with increased % of hedgehogs, it would be difficult to estimate whether the cytotoxic changes are induced by DNA damage or cellular toxic effects. More data are clearly needed to consider how histopathology and hedgehogs can be used in aiding the interpretation of Comet assay results. For the present it would seem prudent to suggest that a positive response in the Comet assay in the presence of histopathological changes or hedgehogs should be interpreted with caution. It may even warrant a repeat assay at lower doses or even a different assay.

45. Finally, based on the above discussion, the VMT concluded that the overall reproducibility and variability of assay results were acceptable among laboratories for negative controls, for the positive control EMS, and for 3 of the 4 coded test chemicals in this step. Further work would be needed with 2-AAF and on the measurement and interpretation of cytotoxicity. It was therefore decided to move to the 2nd step of the 4th phase validation study in order to investigate the predictive capability of *in vivo* Comet assay for chemical carcinogenicity.

9. References

Burlinson B, Tice RR, Speit G, Agurell E, Brendler-Schwaab SY, Collins AR, Escobar P, Honma M, Kumaravel TS, Nakajima M, Sasaki YF, Thybaud V, Uno Y, Vasquez M, Hartmann A. Fourth International Workgroup on Genotoxicity Testing: result of the in vivo comet assay workgroup. Mutat. Res. 2007; 627:31-5.

Hartmann A, Agurell E, Beevers C, Brendler-Schwaab S, Burlinson B, Clay P, Collins A, Smith A, Speit G, Thybaud V, Tice RR. Recommendation for conducting the *in vivo* alkaline comet assay. Mutagenesis 2003; 18:45-51.

ICH Guidance on genotoxicity testing and data interpretation for pharmaceuticals intended for human use S2(R1), ICH harmonized tripartite guideline, 2012

Kirkland D, Speit G. Evaluation of the ability of a battery of three *in vitro* genotoxicity tests to discriminate rodent carcinogens and non-carcinogens III. Appropriate follow-up testing *in vivo*. Mutat. Res. 2008; 654:114-132.

Nakajima M, Ueda M, Yamakage K, Nakagawa Y, Nakagawa M, Ohyama W, Omori T, Asano N, Hayashi M, Uno Y. Tissue sample preparation for *in vivo* rodent alkaline comet assay. Genes Environ. 2012; 34:50-4.

Sasaki YF, Sekihashi K, Izumiyama F, Nishidate E, Saga A, Ishida K, Tsuda S. The comet assay with multiple mouse organs: comparison of comet assay results and carcinogenicity with 208 chemicals selected from the IARC monographs and U.S. NTP Carcinogenicity Database. Crit. Rev. Toxicol. 2000; 30:629-799.

Sekihashi K, Yamamoto A, Matsumura Y, Ueno S, Watanabe-Akanuma M, Kassie F, Knasmüller S, Tsuda S, Sasaki YF. Comparative investigations of multiple organs of mice and rats in the comet assay. Mutat. Res. 2002; 517:53-74.

Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF. Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ. Mol. Mutagen. 2000; 35:206-21.

Phase 4.1 Appendix 1

INTERNATIONAL VALIDATION OF THE *IN VIVO* RODENT ALKALINE COMET ASSAY FOR THE DETECTION OF GENOTOXIC CARCINOGENS (VERSION 14)

Issued by: the Validation Management Team (VMT)
Date: February 6, 2009 revised

A. PURPOSE OF THIS DOCUMENT

This document is provided to clarify the conduct of an international validation study to evaluate the ability of the *in vivo* rodent alkaline Comet assay to identify genotoxic carcinogens, as a potential replacement for the *in vivo* rodent hepatocyte unscheduled DNA synthesis (UDS) assay. A study protocol will be developed by the testing facilities based on the information provided in this document.

B. ASSURANCE OF DATA QUALITY

The study will be conducted in facilities that are Good Laboratory Practice compliant. Consistency between raw data and a final report is the responsibility of each testing facility. The VMT may review the data for consistency, if deemed necessary.

C. ANIMAL WELFARE AND 3Rs

Appropriate national and/or international regulations on animal welfare must be followed. The 3R-principle for experimental animal use must be considered for determining the experimental design.

D.TESTING PROCEDURE

1. MATERIALS AND METHODS

1.1 Test substances and positive/negative controls

1.1.1 Test substance

With the exception of ethyl methanesulfonate (EMS), test substances will be supplied to each testing facility by the VMT. When coded substances are supplied, appropriate safety information will be provided in a sealed envelope to be opened only by an appropriate individual within the organization who is not involved in the study and/or in the case of an emergency. If opened, appropriate documentation and justification will need to be provided to the VMT.

1.1.2 Test substance preparation

Each test substance will be dissolved or suspended with an appropriate solvent/vehicle just before administration (see section 1.1.4.).

1.1.3 Positive control

EMS (CAS No. 62-50-0); the source and lot number to be used will be provided by the VMT. EMS will be dissolved in physiological saline just before administration (within 2 hour).

1.1.4 Negative control (solvent/vehicle)

Solvents/vehicles for test substance preparation will be used as negative controls. An appropriate solvent/vehicle for a test substance may be indicated by the VMT. In the absence of instruction from the VMT, an appropriate solvent/vehicle will be chosen for each test substance by the testing facility in the following order: physiological saline, 0.5% w/v sodium carboxymethylcellulose aqua solution, corn oil.

1.2 Test animals

1.2.1 Species

Although either rats or mice can be used in this assay, the validation study will use rats. The rat is the species most commonly used in toxicological studies and is the preferred species in the *in vivo* rodent hepatocyte UDS assay.

1.2.2 Sex

In order to allow for a direct comparison with the rat hepatocyte UDS assay, males will be used.

1.2.3 Strain

Rat: Crl:CD (SD)

1.2.4 Source

Charles River Laboratories, Inc.

1.2.5 Age

At the time of purchase: 6-8 weeks of age (body weight 150 g - 320 g)

At the time of dosing: 7-9 weeks of age

1.2.6 Body weight

The weight variation of animals should be +/- 20% of the mean weight at the time of dosing.

1.2.7 Number of animals in each dose group at each sampling time

Five males for the validation study. (Notes: we will decide the appropriate number of animals/group afterwards based upon power calculation.)

1.2.8 Animal maintenance

Animals will be reared under appropriate housing and feeding conditions according to the standard operating procedures (SOP) in each testing facility, consistent with Section C "Animal Welfare".

1.2.8.1 Diet

Animals will be fed *ad libitum* with a commercially available pellet diet.

1.2.8.2 Water

Animals will be given free access to tap water *ad libitum* .

1.2.9 Animal quarantine and acclimation

Animals will be quarantined and acclimated for at least 5 days prior to the start of the study, according to SOPs in each testing facility. Only healthy animals approved by the Study Director and/or the Animal Facility Veterinarian will be used.

1.2.10 Animal identification and group assignment

Animals will be identified uniquely and assigned to groups by randomization on the basis of body weight according to the SOP in each testing facility.

1.3 Preparation of Comet assay solutions

The following solutions will be prepared, consistent with laboratory SOPs, unless otherwise specified. (Notes: will likely need to specify shelf life for some solutions as we reconcile lab-specific protocols.)

1.3.1 1.0-1.5% (w/v) standard agarose gel for the bottom layer (if used)

Regular melting agarose will be dissolved at 1.0-1.5% (w/v) in Dulbecco's phosphate buffer (Ca^{++}, Mg^{++} free and phenol free) by heating in a microwave.

1.3.2 0.5 % (w/v) low-melting agarose (Lonza, NuSieve GTG Agarose) gel for the cell-containing layer and, if used, a top layer

Low-melting agarose will be dissolved at 0.5% (w/v) in Dulbecco's phosphate buffer (Ca^{++}, Mg^{++} free and phenol free) by heating in a microwave. During the study this solution will be kept at 37-45°C and discarded afterward.

1.3.3 Lysing solution

The lysing solution will consist of 100 mM EDTA (disodium), 2.5 M sodium chloride, and 10 mM tris hydroxymethyl aminomethane in purified water, with the pH adjusted to 10.0 with 1 M sodium hydroxide and/or hydrochloric acid. This solution may be refrigerated at <10°C until use. On the same day of use, 1 % (v/v) of triton-X100 and 10 % (v/v) DMSO will be added to this solution and the complete lysing solution will be refrigerated at <10°C for at

least 30 minutes prior to use.

1.3.4 Alkaline solution for unwinding and electrophoresis

The alkaline solution consists of 300 mM sodium hydroxide and 1 mM EDTA (disodium) in purified water, pH >13. This solution will be refrigerated at <10°C until use. The pH of the solution will be measured just prior to use.

1.3.5 Neutralization solution

The neutralization solution consists of 0.4 M tris hydroxymethyl aminomethane in purified water, pH 7.5. This solution will be either refrigerated at <10°C or stored consistent with manufacturer's specifications until use.

1.3.6 Mincing buffer

The mincing buffer consists of 20 mM EDTA (disodium) and 10% DMSO in Hank's Balanced Salt Solution (HBSS) (Ca^{++}, Mg^{++} free, and phenol red free if available), pH 7.5 (DMSO will be added immediately before use). This solution will be refrigerated at <10°C until use.

1.3.7 Staining solution

The fluorescent DNA stain is SYBR Gold (Invitrogen-Molecular Probes), prepared and used according to the manufacturer's specifications.

1.4 Comet assay procedure

1.4.1 Experimental design

Compound	Dose (mg/kg)	Number of animals
Vehicle (negative control)	0	5
EMS (positive control)	200	5
Test compound	Low (1/4 of high)	5
Test compound	Medium (1/2 of high)	5
Test compound	High*	5

*High dose selection: in general, in the absence of VMT directions, the high dose level of a test compound will be selected as the dose producing signs of toxicity such that a higher dose level, based on the same dosing regimen, would be expected to produce mortality, or an unacceptable level of animal distress. Selection of doses will be based on the toxicity of the test substance but will not exceed 2000 mg/kg.

1.4.2 Administration to animals

The test substance will be administered three times orally by gavage, 24 and 21 hours apart,

i.e. the second administration is 24 hours after the first administration, and the third administration is 21 hours after the second administration (at 3 hours before animal sacrifice). This regimen will enable us to detect comet and micronucleus at the same time. EMS will be administered once orally by gavage at 3 hours before animal sacrifice. The dosage volume will be 0.1 mL per 10 g body weight in rats on the basis of the animal weight just before administration.

1.4.3 Measurement of body weight and examination of animal conditions

Individual body weights will be measured in accordance with local SOPs and just prior to administration (the weight at this time will be used to determine the volume of each substance administered). The clinical signs of the animals will be observed from just after dosing to just before tissue removal with an appropriate interval according to the SOP in each testing facility.

1.4.4 Tissue sampling

Animals will be humanely killed at 3 hours after second administration of a test substance and at 3 hours after EMS treatment, consistent with Section C "Animal Welfare and 3Rs". The stomach and portions of the liver will be removed. Tissues will be placed into ice-cold mincing buffer, rinsed sufficiently with the cold mincing buffer to remove residual blood (more rinses would likely be needed if exsanguination is not used), and stored on ice until processed. For histopathology, samples will be obtained from the same liver lobe, and from a minimal possible area of stomach.

1.4.5 Preparation of single cells

Single cell preparation should be done within one hour after animal sacrifice. The liver and the stomach will be processed as follows:

Liver: A portion of the left lateral lobe of the liver will be removed and washed in the cold mincing buffer until as much blood as possible has been removed. The size of the portion will be at the discretion of the laboratory but will be standardized. The portion will be minced with a pair of fine scissors to release the cells. The cell suspension will be stored on ice for 15-30 seconds to allow large clumps to settle (or, the cell suspension will be strained through a Cell Strainer to remove lumps and the remaining suspension will be placed on ice), and the supernatant will be used to prepare comet slides.

Stomach: The stomach will be cut open and washed free from food using cold mincing buffer. The forestomach will be removed and discarded. The glandular stomach will be then placed into cold mincing buffer and incubated on ice for from 15 to 30 minutes. After incubation, the surface epithelia will be gently scraped two times using the a scalpel blade or a Teflon scrapper. This layer will be discarded and the gastric mucosa rinsed with the cold

mincing buffer. The stomach epithelia will be carefully scraped 4-5 times (or more, if necessary) with a scalpel blade or Teflon scrapper to release the cells. The cell suspension will be stored on ice for 15-30 seconds to allow large clumps to settle (or, the cell suspension will be strained with a Cell Strainer to remove clumps and the remaining suspension will be placed on ice), and samples of the supernatant used to prepare comet slides.

1.4.6 Slide preparation

Slide preparation should be done within one hour after single cell preparation. Comet slides will be prepared using laboratory specific procedures. The volume of the cell suspension added to 0.50% low melting agarose to make the slides will not decrease the percentage of low melting agarose by more than 10% (i.e., not below 0.45%) .

1.4.7 Lyses

Once prepared, the slides will be immersed in chilled lysing solution overnight in a refrigerator under a light proof condition. After completion of lysing, the slides will be rinsed in purified water or neutralization solution to remove residual detergent and salts prior to the alkali unwinding step.

1.4.8. Unwinding and electrophoresis

Slides will be randomly placed onto a platform of submarine-type electrophoresis unit and the electrophoresis solution added. A balanced design will be used (i.e., in each electrophoresis run, there should be the same number of slides from each animal in the study; see Attachment 1, an example of use to keep track of each slides during each electrophoresis run. Each laboratory will need to provide its own electrophoresis box chart, as different boxes can accommodate different numbers of slides). The electrophoresis solution will be poured until the surfaces of the slides are completely covered with the solution. The slides will be left to be unwind for 20 minutes. Next, the slides will be electrophoresed at 0.7 to 1 V/cm (Notes: the voltage may be defined more strictly, e.g. 0.7 exactly, based on the 3rd phase validation study results), with a constant voltage at approximately 0.30 A. The current at the start and end of the electrophoresis period should be recorded. The temperature of the electrophoresis solution through unwinding and electrophoresis should be maintained at a constant temperature <10°C . The temperature of the electrophoresis solution at the start of unwinding, the start of electrophoresis, and the end of electrophoresis should be recorded. The electrophoresis duration should result in an average DNA migration in the negative control group of 1-8% DNA in the tail for the liver, and 1-30% (preferably 1-20%) DNA in the tail for the stomach.

1.4.9. Neutralization and dehydration of slides

After completion of electrophoresis, the slides will be immersed in the neutralization buffer for at least 5 minutes. All slides will be dehydrated by immersion into absolute ethanol

(≥99.6%) for at least 5 minutes if slides will not be scored soon, allowed to air dry, and then stored until scored at room temperature, protected from humidity > 60 %. Once scored, slides should be retained and stored under low humidity conditions (e.g., in a desiccator) for potential rescoring.

1.4.10. DNA staining, comet visualization and analysis

Coded slides will be blind scored according to laboratory specific SOPs. The slides will be stained with SYBR Gold according to manufacturer's specifications. The comets will be measured via a digital (e.g. CCD) camera linked to an image analyzer system using a fluorescence microscope at magnification of 200X. For each sample (animal/tissue), fifty comets cells per slide will be analyzed, with 2 slides scored per sample (Notes: to be re-evaluated after statistical analysis). Approximately 10 areas/slide should be observed at 5 cells or less/field (may require dilution of cell suspension during the single cell preparation process), taking care to avoid any selection bias, overlap counting of cells, and edge areas of slides. Heavily damaged cells exhibiting a microscopic image (commonly referred to as hedgehogs) consisting of small or non-existent head and large, diffuse tails will be excluded from data collection if the image analysis system can not properly score them (Add pictures in an appendix – indicate if scorable by software then should be scored). However, the frequency of such comets should be determined per sample, based on the visual scoring of 100 cells per sample. The comet endpoints collected will be % tail DNA, tail length in microns measured from the estimated edge of the head region closest to the anode, and, if possible for a particular image analysis system, Olive tail moment [= a measure of tail length (a distance between a center of head mass and a center of tail mass; microns) X a measure of DNA in tail (% tail DNA/100): Olive et al., 1990]. (Notes: at Atagawa meeting held on March 13-14, 2008, there were some discussions about necessity of tail length and Olive tail moment. As a tentative consensus, these parameters are no longer necessary to analyze statistically in this validation effort, because %DNA in tail seems a sufficient endpoint for validation. But data on tail length and tail moment will be collected to prepare for the future analysis)

1.4.11. Histopathology

When a positive Comet assay response is obtained for a tissue, a sample histopathological assessment will be conducted to evaluate for the presence of examined for the tissue according to the SOP in each testing facility.

2 STATISTICS

Different approaches for data analysis have been proposed for comet data generated across a range of test substance dose levels (Lovell et al. 1999; Hartmann et al. 2003; Wiklund and

Agurell 2003). The primary endpoint of interest for DNA migration is the % tail DNA. In addition, the distribution of migration patterns among cells within an animal will be considered. The percentage of "hedgehogs" and of cells with low molecular weight DNA will also be evaluated as a function of treatment. The unit of analysis for a specific tissue is the individual animal. Each laboratory may make their own conclusion about the *in vivo* genotoxicity of a test substance using their standard approach.

In data analysis process of this validation study, three conceptual key terms, i.e. "Endpoint", "Estimate", and "Effect" are defined and used. Briefly, "Endpoint" is defined as individual observed values for a parameter such as % DNA in tail. "Estimate" is defined as a mean or median calculated with values of a particular "Endpoint" in each animal. "Effect" is defined as difference or ratio of an average of "Estimate" between a negative control group and a treatment group. A general purpose in data analysis of validation studies is to investigate how large variation exists among data from testing facilities, and "Effect" is considered as a good yardstick (criterion) to understand the variation of Comet parameters among testing facilities. Thus "Effect" will be used in this validation study. Dunnett's one side test is also applied for data analysis.

3 DATA AND REPORTING

3.1 Treatment of results

Individual animal data and group summaries will be presented in a fixed tabular form that will be provided from the VMT.

3.2 Evaluation and interpretation of results

A positive response is defined as a statistically significant change in the % tail DNA in at least one dose group at a single sampling time in comparison with the negative control value. The positive control should produce a positive response, and if not, the study data will not be acceptable. Where a positive response is obtained in a test substance group, the investigator(s) will assess the possibility that a cytotoxic rather than a genotoxic effect is responsible based on the percentage of cells with low molecular weight DNA and histopathology. Positive results indicate that the test substance induce DNA damage in the target tissue(s) investigated. Negative results indicate that, under the test conditions used, the test substance does not induce DNA damage *in vivo* in the tissue(s) evaluated.

3.3 Study report

The study report from each testing facility will at least include the following information:

3.3.1 Test substance and positive/negative controls

Identification; CAS number; supplier; lot number; physical nature and purity; physiochemical property relevant to the conduct of the study, if known; justification for choice of vehicle; and solubility and stability of the substances in the solvent/vehicle, if known.

3.3.2 Test animals

Species/strain used; number, age and sex of animals; source, housing conditions, quarantine and acclimation procedure, and animal identification and group assignment procedure; individual weight of the animals on the day of receipt, at the end of the acclimation period, and before administration (at the time of grouping), including body weight range, mean and standard deviation for each group; and choice of tissue(s) and justification.

3.3.3 Reagents to prepare reagent solutions

Identification; supplier; lot number; and time limit for usage if known.

3.3.4 Test conditions

Data from range-finding study, if conducted; rationale for dose level selection; details of test substance preparation; details of the administration of the test substance; rationale for route of administration; methods for verifying that the test substance reached the general circulation or target tissue, if applicable; details of food and water quality; detailed description of treatment and sampling schedules; method of measurement of toxicity, including histopathology; detailed methods of single cell preparation; method of slide preparation, including agarose concentration, lysis conditions, alkali conditions and pH, alkali unwinding time and temperature, electrophoresis conditions (pH, V/cm, mA, and temperature at the start of unwinding and the start and the end of electrophoresis) and staining procedure; criteria for scoring comets and number of comets analyzed per slide, per tissue and per animal; evaluation criteria; criteria for considering studies as positive, negative or equivocal.

3.3.5 Results

Signs of toxicity, including histopathology in the appropriate tissue(s) if applicable; individual and mean/median values for DNA migration (and ranges) and % cells with low molecular weight DNA and % hedgehogs in individual tissue, animal, and group; concurrent positive and negative control data; and statistical evaluation.

3.3.6 Discussion of the results and/or conclusion, as appropriate.

4 ARCHIVES AND REVIEW

The study report and all raw data (including slide samples and image data) from this study will be retained according to the SOP in each testing facility. All raw data will be submitted to the management team for review if required.

5 REFERENCES

Burlinson B, et al., 4th International Workgroup on Genotoxicity Testing: result of the in vivo comet assay workgroup (in preparation).

Collins AR, et al., Direct enzymatic detection of endogenous oxidative base damage in human lymphocyte DNA. Carcinogenesis, 14, 1733-1735, 1993.

Hartmann A, et al., Recommendation for conducting the *in vivo* alkaline Comet assay. Mutagenesis, 18(1), 45-51, 2003.

Lovell DP, G Thomas G, R Dubow., Issues related to the experimental design and subsequent statistical analysis of in vivo and in vitro comet studies. Teratog Carcinog Mutagen. 19(2), 109-119, 1999.

Olive PL, et al., Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cell using the "comet" assay. Radiat. Res., 122, 86-94, 1990.

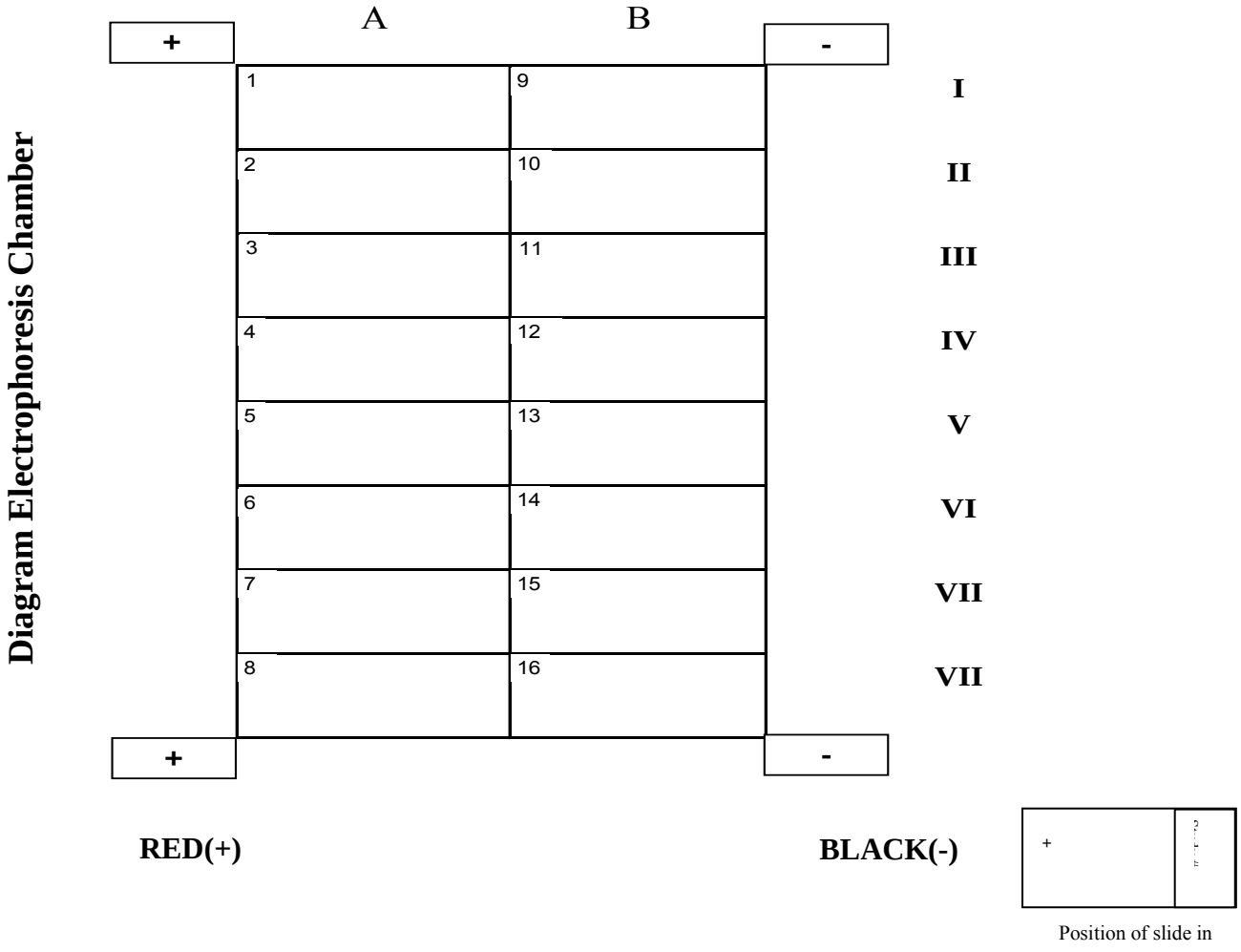
Tice RR et al., Single cell gel/Comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ. Mol. Mutagen., 35, 206-221, 2000.

Wiklund SJ, E Agurell., Aspects of design and statistical analysis in the Comet assay. Mutagenesis 18(2):167-175, 2003.

Attachment 1:

SLIDES UNWINDING & ELECTROPHORESIS RECORDING SHEET

Electrophoresis Run #				Initials & Date
Approximate alkaline electrophoresis buffer volume in chamber				
Unwinding				
Time	Total	Start	End	
Buffer Temperature				
Electrophoresis				
Running time	Total	Start	End	
Volts				
Milliamperes				
Buffer Temperature				
Thermometer No.				
Electrophoresis chamber No.				
Power supply No.				



Phase 4.1 Appendix 2

INTERNATIONAL VALIDATION OF THE *IN VIVO* RODENT ALKALINE COMET ASSAY FOR THE DETECTION OF GENOTOXIC CARCINOGENS
- Study Plan for 1st Step of 4th Phase Validation Study -

Issued by: the Validation Management Team (VMT)
Date: May 10, 2009

PURPOSE OF THIS DOCUMENT

This document is provided as a supplement to the study protocol to clarify the purpose, schedule, and specific notes of each trial of an international validation study to evaluate the ability of the *in vivo* rodent alkaline Comet assay.

STUDY TITLE

1st step of 4th phase validation study of international validation of the *in vivo* rodent alkaline Comet assay for the detection of genotoxic carcinogens (abbreviation: 1st step of 4th phase validation study of *in vivo* Comet assay)

BACKGROUND AND PURPOSE OF THIS STUDY

In the 3rd phase of the *in vivo* Comet assay validation study, three coded test compounds and EMS, a positive control, were assayed in four leading laboratories in accordance with the Comet assay protocol-version 13, and generally comparable data were obtained. To obtain more consistent data between laboratories, the protocol was further optimized and revised to version 14. The version 14 protocol additionally involves 3 treatments (0, 24, and 45 h) before collecting liver and glandular stomach tissue samples at 48 h after the first treatment. This allows for the detection of DNA damage using the Comet assay and of micronuclei in blood erythrocyte using the standard micronucleus assay in the same animals, which will significantly reduce the use of animals for in vivo genotoxicity testing.

In the 1st step of the 4th phase of this validation study, the purpose is to examine the extent of reproducibility and variability of assay results among laboratories using coded test chemicals and the positive control EMS, when experiments are conducted in accordance with the Comet assay protocol-version 14. After reviewing data from this study, the VMT will decide whether or not the 2nd step of 4th phase validation study can be started with an expanded set of test chemicals.

SCHEDULE

May 10, 2009: Agreement of the study plan by the VMT

~May 20, 2009: Delivery of protocol-version 14 and study plan to testing facilities

~May 31, 2009: Delivery of one test chemical

~June 30, 2009: Delivery of data-spread sheet (Excel file)

June ~ August, 2009: Experimental period (the experiment should be started after acceptance of the study protocol and preparation of appropriate SOPs in each testing facility; the inputted data-spread sheet should be submitted to VMT soon after the data are available)

August 25-26, 2009: Meeting on Comet international validation study at Firenze, Italy (data will be presented by each laboratory, if possible)

September 30, 2009: Deadline of all data submission to VMT

~October 31, 2009: Limited data analysis and decision-making by VMT to start the 2nd step of 4th phase validation study with the expanded set of chemicals

~December 31, 2009: Finalization of data analysis

SPECIFIC NOTES

SUCCESS CRITERIA

To obtain positive results in all positive control groups in all testing facilities.

To obtain consistent positive or negative results in testing facilities that examine the same test chemical.

OTHERS

Dose selection of coded test chemical

The dose levels of coded test chemical will be decided by each facility. The VMT may provide some toxicological information such as LD50 about the coded test chemical to assist in this process.

Solvent/vehicle

The solvent/vehicle to prepare the dosing formulation of coded test chemical will be decided by each facility.

Comet visualization and analysis

The VMT and Consultation team will send a color atlas for reference to distinguish between comets and hedgehogs to each facility by the end of May, 2009. Each facility should base their decision criteria on this color atlas.

Title: Report of the JaCVAM initiative international validation study of the *in vivo* rodent alkaline Comet assay for the detection of genotoxic carcinogens: the 4th (definitive) phase-2nd step

Issued: Yoshifumi Uno, D.V.M., Ph.D., and a Validation Management Team (VMT) member

Notes: this document was prepared to summarize the *in vivo* Comet assay validation process and results in the 4th (definitive) phase-2nd step. The methods are mentioned minimally in this document, because the details are described in the study protocol and the study plan (Appendices 1 and 2).

1. Introduction

1. The *in vivo* rodent alkaline Comet assay is used worldwide for detecting DNA damage as evidenced by strand breaks. The assay can be applied to the investigation of genotoxic potential of test chemicals, and is currently identified as a second *in vivo* genotoxicity assay in the ICH-S2(R1) guidance (2012) along with the more usual *in vivo* micronucleus test in bone marrow or peripheral blood. The Comet assay protocol has been discussed in the meetings of the International Workshops on Genotoxicity Testing (IWGT) and the International Comet Assay Workshop (ICAW), and consensus articles have been published (Tice, et al., 2000, Hartmann, et al., 2003, Burlinson, et al., 2007).

2. The assay, however, has not been validated formally with a standardized study protocol. In addition, since reports on the predictive capability of the *in vivo* rodent Comet assay for carcinogenicity are limited (Sasaki, et al., 2000, Sekihashi, et al., 2002, Kirkland, et al., 2008a), the investigation of predictive capability in multiple laboratories using one validated study protocol would be more useful to understand the overall performance of the assay. The Japanese Environmental Mutagen Society/the Mammalian Mutagenicity Study Group (JEMS/MMS) decided to have an (international) collaborative study of the *in vivo* Comet assay in 2003, and conducted a preliminary collaborative study on Comet assay procedure, e.g., comparison of assay results between whole cells and isolated nuclei (Nakajima, et al., 2012). At the same time other groups of scientists wished to establish an OECD guideline for the Comet assay. A coordinated validation effort for the *in vivo* Comet assay was therefore required, and so the Japanese Center for the Validation of Alternative Methods (JaCVAM) organized an international validation study, commencing April, 2006, in cooperation with the U.S. National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), the European Centre for the Validation of Alternative Methods (ECVAM), and JEMS/MMS.

3. The purpose of this validation study was to evaluate the ability of the *in vivo* Comet assay to identify genotoxic chemicals as a potential predictor of rodent carcinogenicity, and as an alternative follow-up assay to more the commonly used *in vivo* rodent Unscheduled DNA Synthesis (UDS) assay. The ultimate goal of this validation effort is to establish an OECD guideline for the *in vivo* rodent alkaline Comet assay.

2. Background and Purpose

4. In the 1st step of the 4th phase validation study (Phase 4-1), the purpose was to examine the extent of reproducibility and variability of the *in vivo* Comet assay results among laboratories using a small number of coded test chemicals and the positive control ethyl methanesulfonate

(EMS), when experiments were conducted in accordance with the Comet assay protocol-version 14. In the review of study data, the Validation Management Team (VMT) confirmed the acceptable overall reproducibility and variability of assay results among laboratories for the positive control and 3 of the 4 coded chemicals. Thus the VMT decided to move onto the 2nd step of 4th phase validation study (Phase 4-2) with an expanded set of test chemicals. However, due to continuing concerns with the detection of 2-AAF (the 4th of the coded chemicals in Phase 4-1) and issues regarding cytotoxicity, the Comet assay protocol was further modified to version 14.2 (Appendix 1) for the Phase 4-2 studies.

5. The purpose of Phase 4-2 was to investigate the predictive capability of the assay relative to the carcinogenicity of selected test chemicals and taking into account expectations of effect based on existing genotoxicity and other data and, information on mode of action (see the study plan: Appendix 2).

3. Experimental Period

December, 2009 - February, 2012

4. Participant Laboratories

6. Fourteen laboratories* participated in the Phase 4-2 validation study. These included four leading laboratories# that were very experienced in the Comet assay and participated in the first three phases of the pre-validation studies, plus ten laboratories that passed the recruitment process for this 4th phase validation study.

* Merck Research Laboratories# (code: Lab B), BioReliance# (Lab C), Huntingdon Life Sciences# (Lab D), Food and Drug Safety Center# (Lab E), The Institute of Environmental Toxicology (Lab F), Novartis Pharma (Lab G), AstraZeneca (Lab H), Sumitomo Chemical (Lab I), Mitsubishi Chemical Medience (Lab J), Janssen R&D (Lab K), Health Canada (Lab L), Covance (Lab M), Bayer Schering Pharma (Lab N), and Integrated Laboratory System (Lab O).

5. Success Criteria in the Study Plan (Appendix 2)

7. To obtain the predictive capability (values of positive sensitivity and negative specificity) of the assay in relation to the carcinogenicity of the test chemicals, the VMT discussed at a meeting held at Salt Lake on March 12, 2010, whether or not expected positive sensitivity and negative specificity should be set as success criteria before starting this step of validation study. The VMT concluded that it was unnecessary because such values would be calculated resultantly after the validation study. However, these data will be disclosed and can be used for further analysis by anyone who wants to re-evaluate. Although it was a primary objective to determine the sensitivity and specificity of the rodent *in vivo* Comet assay in relation to the known carcinogenicity of the chemicals, it was also considered relevant to take into account species-specific and tissue specific carcinogenic effects that may not be detected in the rat or in

the tissues selected for the validation exercise (namely glandular stomach and liver), route of administration that may not be optimal, and also modes of action that may not give rise directly to DNA damage (e.g. aneuploidy). Therefore, a supplementary analysis of the expected Comet assay outcomes in the stomach and liver of rats for the known genotoxic carcinogens and non-carcinogens was conducted after the trial was completed, and this supplementary analysis is also included in the Discussion.

6. Materials and Methods

8. In this section an outline of the materials and methods is described. The details are referred to in the validation study protocol version 14.2 (Appendix 1), and the study reports written by each laboratory.

9. An individual study protocol was prepared in each laboratory in accordance with the validation study protocol v.14.2. The experiments proceeded in each facility based on their own study protocol and SOPs.

6-1. Animal species, strain, and sex

10. Rats were selected in this validation effort because they are the most popular species in toxicology studies. Crl:CD(SD) male rats were used.

6-2. Test chemicals, vehicles, and dose levels

11. Forty coded test chemicals were used in this study (Table 1). The reasons for selection of those test chemicals are described in the chemical selection report written by Dr. Takeshi Morita (Appendix 3). Briefly, test chemicals were selected from four categories based on their genotoxicity and carcinogenicity properties, i.e., genotoxic carcinogens, genotoxic non-carcinogens, non-genotoxic carcinogens, and non-genotoxic non-carcinogens. Genotoxicity was defined as a positive result in the Ames test or in standard *in vivo* genotoxicity tests such as the bone-marrow micronucleus assay. Results of *in vitro* genotoxicity assays using mammalian cells were not always considered as relevant for test chemical selection, since mammalian cell genotoxicity assays often produce irrelevant positive results for *in vivo* genotoxicity and/or carcinogenicity (Kirkland, et al., 2005). In consideration of this background, non-genotoxic non-carcinogens used in this validation study included four chemicals considered to produce irrelevant positive results in mammalian cell genotoxicity assays, i.e., *o*-anthranilic acid, ethionamide, isobutyraldehyde and *t*-butylhydroquinone. These are listed as the so-called "false-positive" chemicals in the recommended list of genotoxic and non-genotoxic chemicals for assessment of the performance of new or improved genotoxicity tests (Kirkland, et al., 2008b). Carcinogenicity was defined as positive results in rodent (rat and/or mouse) carcinogenicity studies, or known human carcinogens.

12. The test chemicals included organic and inorganic chemicals with various modes of action for genotoxicity and/or carcinogenicity, e.g., base-alkylation, aneugenic effects, bulky adduct

formation, formation of cross-links, epoxide formation, nucleoside analogue, cytotoxicity, and peroxisome proliferation.

13. The test chemicals were randomly coded by JaCVAM (Table 1), and sent to an assigned chemical master of each testing facility, who was independent of the validation study. Since the VMT considered that the intra- and inter-laboratory reproducibility of the assay had already been demonstrated amongst the participating laboratories using the four coded test chemicals and the positive control EMS in Phase 4-1, each of the 40 coded test chemicals in Phase 4-2 was evaluated in one laboratory (Table 1). The vehicle for each coded test chemical was appropriately selected in each testing facility (Table 1). The VMT provided some toxicological information such as the LD_{50}, if available from the published literature, in order to assist the dose selection process in each laboratory. However, for most chemicals the dose levels were decided in each facility based on the results of preliminary dose range-finding studies designed in each laboratory. The exceptions to this process were the selection of dose levels and vehicles by the VMT for three chemicals, *N*-nitrosodimethylamine, 1,2-dimethylhydrazine and sodium arsenite, which needed to be retested in another laboratory (Table 1).

Table 1 Test chemical code, test chemical name, category, laboratory tested, vehicle, and dose levels

Test chemical code	Test chemical name (CASRN)	Category of genotoxicity and carcinogenicity	Lab tested (coded lab name)	Vehicle	Dose level (mg/kg/day)
A4114	2-Acetylaminofluorene (53-96-3)	Genotoxic carcinogen	Lab O	Corn oil	250, 500, 1000
A4201	1,3-Dichloropropene (542-75-6)	Genotoxic carcinogen	Lab C	Corn oil	50, 100, 200
A4202	Ethionamide (536-33-4)	Non-genotoxic non-carcinogen	Lab C	Corn oil	125, 250, 500
A4203	Busulfan (55-98-1)	Genotoxic carcinogen	Lab C	Corn oil	10, 20, 40
A4204	*N*-Nitrosodimethylamine (62-75-9)	Genotoxic carcinogen	Lab L	Saline	2.5, 5, 10
			Lab O *	Saline *	0.63, 1.25, 2.5 *
A4205	Ampicillin trihydrate (7177-48-2)	Non-genotoxic non-carcinogen	Lab L	Saline	25, 50, 100
				Corn oil	500, 1000, 2000
A4206	1,2-Dimethylhydrazine dihydrochloride (306-37-6)	Genotoxic carcinogen	Lab L	Saline	6.25, 12.5, 25
			Lab O *	Saline *	1.56, 3.13, 6.25 *
A4207	Isobutyraldehyde (78-84-2)	Non-genotoxic non-carcinogen	Lab B	Corn oil	500, 1000, 2000
A4208	Cisplatin	Genotoxic	Lab B	0.5% CMC	6, 12.5, 25

	(15663-27-1)	carcinogen			
A4209	Azidothymidine (30516-87-1)	Genotoxic carcinogen	Lab B	0.5% CMC	500, 1000, 2000
A4210	*p*-Chloroaniline (106-47-8)	Genotoxic carcinogen	Lab D	Corn oil	37.5, 75, 150
Test chemical code	Test chemical name (CASRN)	Category of genotoxicity and carcinogenicity	Lab tested (coded lab name)	Vehicle	Dose level (mg/kg/day)
A4211	*t*-Butylhydroquinone (1948-33-0)	Non-genotoxic non-carcinogen	Lab D	Corn oil	131.3, 262.5, 525
A4212	Methyl carbamate (598-55-0)	Non-genotoxic carcinogen	Lab D	Saline	500, 1000, 2000
A4213	Methyl methanesulfonate (66-27-3)	Genotoxic carcinogen	Lab G	Saline	20, 40, 80
A4214	2,6-Diaminotoluene (823-40-5)	Genotoxic non-carcinogen	Lab G	Corn oil	150, 300, 600
A4215	5-Fluorouracil (51-21-8)	Genotoxic non-carcinogen	Lab G	Saline	25, 50, 100
A4216	8-Hydroxyquinoline (148-24-3)	Genotoxic non-carcinogen	Lab N	Corn oil	125, 250, 500
A4217	Hydroquinone (123-31-9)	Genotoxic carcinogen	Lab N	Saline	125, 250, 500 [1)]
A4218	Saccharin (81-07-2)	Non-genotoxic carcinogen	Lab N	Corn oil	500, 1000, 2000
A4219	Sodium arsenite (7784-46-5)	Genotoxic carcinogen	Lab M	Saline	7.5, 15, 30
			Lab O *	Saline *	7.5, 15, 30 *
A4220	Thioacetamide (62-55-5)	Non-genotoxic carcinogen	Lab M	Saline	19, 38. 75
A4221	Diethanolamine (111-42-2)	Non-genotoxic carcinogen	Lab M	Saline	175, 350, 700
A4222	*p*-Phenylenediamine dihydrochloride (624-18-0)	Genotoxic non-carcinogen	Lab K	Saline	25, 50, 100
A4223	*o*-Phenylphenol sodium salt (132-27-4)	Non-genotoxic carcinogen	Lab K	Corn oil	250, 500, 1000
A4224	2,4-Diaminotoluene (95-80-7)	Genotoxic carcinogen	Lab K	Saline	100, 150, 200
A4225	4,4′-Oxydianiline	Genotoxic	Lab H	0.5% CMC	50, 100, 200

	(101-80-4)	carcinogen			
A4226	*o*-Anisidine (90-04-0)	Genotoxic carcinogen	Lab O	Corn oil	150, 300, 600
A4227	Sodium chloride (7647-14-5)	Non-genotoxic non-carcinogen	Lab O	Water	500, 1000, 2000
A4228	Acrylonitrile (107-13-1)	Genotoxic carcinogen	Lab E	Corn oil	15.7, 31.3, 62.5
Test chemical code	Test chemical name (CASRN)	Category of genotoxicity and carcinogenicity	Lab tested (coded lab name)	Vehicle	Dose level (mg/kg/day)
A4229	9-Aminoacridine hydrochloride monohydrate (52417-22-8)	Genotoxic non-carcinogen	Lab E	Corn oil	15.7, 31.3, 62.5
A4230	Ethanol (64-17-5)	Non-genotoxic carcinogen	Lab E	Saline	500, 1000, 2000
A4231	1,2-Dibromomethane (106-93-4)	Genotoxic carcinogen	Lab J	Corn oil	25, 50, 100
A4232	*p*-Anisidine (104-94-9)	Genotoxic non-carcinogen	Lab J	0.5% CMC	125, 250, 500
A4233	*o*-Anthranilic acid (118-92-3)	Non-genotoxic non-carcinogen	Lab J	0.5% CMC	500, 1000, 2000
A4234	Benzene (71-43-2)	Genotoxic carcinogen	Lab I	Corn oil	500, 1000, 2000
A4235	Di(2-ethylhexyl)phthalate (117-81-7)	Non-genotoxic carcinogen	Lab I	Corn oil	500, 1000, 2000
A4236	Trisodium EDTA monohydrate (10378-22-0)	Non-genotoxic non-carcinogen	Lab I	Saline	500, 1000, 2000
A4237	Cadmium chloride (10108-64-2)	Genotoxic carcinogen	Lab F	Saline	20, 40, 80
A4238	Chloroform (67-66-3)	Non-genotoxic carcinogen	Lab F	Corn oil	125, 250, 500
A4239	D,L-Menthol (15356-70-4)	Non-genotoxic non-carcinogen	Lab F	Corn oil	125, 250, 500

* The vehicle and dose levels were directed by VMT, because those chemicals were retested in another laboratory due to the reasons described in the section 7-2.

6-3. Positive control

14. EMS was used as a positive control in this validation, because it is a well-known genotoxic chemical for multiple organs, and had been shown to give reproducible responses in the

pre-validation studies and in Phase 4-1. EMS was dissolved in physiological saline, and administered orally by gavage to rats at the dose level of 200 mg/kg twice (21 hr interval).

6-4. VMT consensus about expected assay results for test chemicals

15. It would be necessary to have expected assay results for each category of four classes of test chemicals (i.e., genotoxic carcinogen, genotoxic non-carcinogen, non-genotoxic carcinogen, and non-genotoxic non-carcinogen) before review of assay results in order to avoid any bias for the evaluation of assay results. VMT discussed and decided expected assay results at Salt Lake meeting held on March 12, 2010, as follows.

6-4-1. Genotoxic carcinogen: positive results will be expected in the liver and/or the stomach. Target organ specificity of carcinogenicity may be considered for the interpretation of negative results, but it should be minimized because our validation study protocol is designed for screening purpose against carcinogenicity without consideration of the target organ specificity.

6-4-2. Genotoxic non-carcinogen: negative results will be preferred in both the liver and the stomach, but positive results will be acceptable because this category of chemicals is considered to have genotoxic activity essentially.

6-4-3. Non-genotoxic carcinogen: negative results will be preferred in both the liver and the stomach, but positive results will be acceptable because this category of chemicals may have some genotoxic mode of action for carcinogenicity. But, in case of positive response of known cytotoxic agents, more careful consideration will be required for the interpretation of positive results.

6-4-4. Non-genotoxic non-carcinogen: negative results will be expected.

16. The above expectations were not disclosed to the participating laboratories prior to testing.

17. Although the above were defined as the pre-trial expectations (i.e. based mainly on carcinogenic properties), as mentioned above, it was subsequently considered useful and important to consider species-specific and tissue specific effects, route of administration and mode of action, and to thereby predict the outcome of the Comet assay in rat liver and stomach for these chemicals, irrespective of the overall carcinogenicity. Thus, a supplementary analysis was performed, taking such factors into account, and is included in the Discussion.

6-5. Administration of test chemical to animals

18. Each coded test chemical was administered to rats at three dose levels, on three occasions (0, 24 and 45 hours, i.e. with 24 and 21 hours intervals between the doses) by oral gavage. This administration regimen was designed to allow the combination of micronucleus and Comet endpoints into a single assay, in consideration of the 3R's principle for animal use. Investigation into micronucleus induction was optional and micronucleus data are not included in this

validation study (micronucleus data may be included in study reports written by testing facilities).

6-6. Organs analyzed

19. The liver and the stomach (glandular stomach) were selected in this validation effort, because the former is the primary organ for the metabolism of absorbed chemicals, and the latter is a site of first contact of chemicals after oral administration. These organs were recommended for screening of genotoxic chemicals in the previous discussion in ICAW (Hartmann, et al., 2003).

6-7. Data-acceptance criteria

20. Data-acceptance criteria were determined based on the 1st to 3rd phase pre-validation study results. The criteria for data-acceptance were:

6-7-1. Negative (vehicle) control

Means of %DNA in tail are 1-8% in the liver and 1-30% (preferably 1-20%) in the stomach.

6-7-2. Positive control (EMS)

Effect (difference of means of % DNA in tail between EMS and vehicle control groups) must show a statistically significant increase (see the section 6-8.) and be 5% or higher in the liver and the stomach. This section 6-7-2. is the primary criterion for data-acceptance.

6-8. Data analysis of % DNA in tail

21. % DNA in tail was used as the primary endpoint of this validation study, because it is considered linearly related to the DNA break frequency over a wide range of DNA damage levels (Hartmann, et al., 2003). Other parameters such as tail moment and tail length may be calculated and reported in the study reports written by the testing facilities, but no statistical analysis was applied to such parameters in this validation study report.

22. Three conceptual key terms, “Endpoint”, “Estimate” and “Effect” were defined and used in the data analysis of this validation study. Briefly, Endpoint is defined as individual observed values for a parameter such as % DNA in tail. Estimate is defined as a mean calculated with values of the Endpoint in each animal. Effect is defined as the difference (hereafter designated as Effect (diff.)) or ratio (hereafter designated as Effect (ratio)) of a mean of an Estimate between a negative control group and a treatment group. The general purpose of data analysis in validation studies is to investigate how large is the variation that exists among data from several testing facilities, and Effect is considered as a good indicator to understand the variation of comet assay parameters among testing facilities. VMT noticed through the 1st to the 3rd phase pre-validation studies that Effect (diff.) was more useful for the comparison of variation than Effect (ratio), because Effect (ratio) depended on the magnitude of the negative control values (i.e. lower negative control values easily produced higher Effect (ratio)) and would be often misleading in the evaluation of responses induced following a test chemical administration.

Therefore Effect (diff.) was used to evaluate the assay results.

23. Dunnett's test (two-sided, $P<0.05$) and the linear Trend test (two-sided, $P<0.05$) were applied to Effect (diff.) in the groups of coded test chemicals. The two-sided analysis was used because both increases and decreases in the comet parameter could be detected, although decreases could be detected more easily in the stomach where control % tail DNA levels were higher than in the liver. A decreased % DNA in tail was expected to be a good index to detect cross-linking agents. For the positive control group, Student's t-test (one-sided, $P<0.025$) was applied to the Effect (diff.).

6-9. Histopathology

24. Regarding the evaluation of cytotoxicity, the "Gold Standard" for assessing levels of necrosis and apoptosis when an *in vivo* Comet assay was positive was concluded to be histopathology. This was a consensus recommendation of the IWGT meeting in 2005 (Burlinson, et al., 2007), where it was also pointed out that there was a need to standardize ways to present histopathological findings. In this validation study, when increased % DNA in tail was observed in the liver and/or stomach, the organ(s) was histopathologically examined by pathologists of the testing laboratory, except for retests of *N*-nitrosodimethylamine, 1,2-dimethylhydrazine and sodium arsenite, which were examined by pathologists in Biosafety Research Center (BSRC). The reason why the histopathological effects of those three chemicals were examined in laboratories other than those that performed the Comet assays was as follows. At the time of the retests in Lab O, BSRC planned independent re-examination of the histopathology of tissue samples from the animals treated with test chemicals, if the samples were available. In addition, Lab O asked BSRC to examine the histopathology on their behalf. The VMT and BSRC accepted this proposal (note: the BSRC examination was independent from this validation study, but the report is available for review. See the report of "Evaluation of pathological specimens obtained from Comet assay validation studies" (Appendix 4).

25. Based on the IWGT consensus recommendations (Burlinson, et al., 2007), the VMT decided that histopathology results should primarily be considered to interpret any relationship between positive findings in the Comet assay and the cytotoxic effects of the test chemicals. Increased % hedgehog cells was also under consideration for cytotoxicity evaluation, but it was not adopted in this validation study. However, the data were collected because increased % hedgehog cells may be useful to indicate not only cellular toxicity but also DNA damage.

26. In histopathological examination, the VMT considered that necrosis and/or degeneration (or findings indicating degenerative changes) would be the main indicators of cellular toxicity. In contrast, apoptosis could indicate both cellular toxicity and DNA damage.

In deciding standardized ways to present histopathological findings, the VMT firstly reviewed histopathological data of test chemicals showing negative results in Comet assay. For most of

the negative compounds, there seemed to be no histopathological findings indicating cytotoxicity related to chemical treatment, except for the following cases.

1) 5-Fluorouracil: single cell necrosis in glandular stomach mucosa (grade: minimal; incidence: two of five (2/5) rats at 25 mg/kg, and 5/5 rats at 50 and 100 mg/kg)
2) Hydroquinone: single cell necrosis in hepatocytes (minimal to slight; 5/5 rats at 500 mg/kg)
3) Di(2-ethylhexyl)phthalate: eosinophilic degeneration/necrosis in hepatocytes (slight; 5/5 rats at 2000 mg/kg)
4) Histopathology of the stomach with many chemicals (e.g., *o*-phenylphenol sodium salt, *p*-anisidine, *o*-anthranilic acid) showed ulcer/erosion in the glandular stomach or forestomach, and the grade of lesions was minimal to slight (or mild). Since these findings are often observed in rats, it is thought to be caused by stress (secondary changes), and may not always indicate cytotoxicity.

27. Since the above chemicals all gave negative comet results, these findings indicate that minimal to slight (or mild) histopathological changes related to cytotoxicity would not affect increased % DNA in tail in the liver and the stomach. Therefore, the VMT decided that both the grade and the incidence of the histopathological changes should be considered in the interpretation of cytotoxic effects in relation to Comet assay responses. For example, when one of five animals showed minimal to slight (or mild) necrosis and/or degenerative findings in a target organ, such weak changes would not be expected to affect the Comet assay results. In addition, the study directors and/or pathologists in most of the participant laboratories considered that minimal to slight (or mild) single cell necrosis or single cell death would not affect the Comet assay results. The VMT agreed with this interpretation. On the other hand, moderate or severe necrosis in the target organ could be considered sufficient to affect the Comet assay results.

28. The above criteria were presented to participants at the Kyoto meeting held on September 12-13, 2011, and accepted. Based on those criteria, the VMT and the meeting-participants interpreted the significance of positive findings in the Comet assay under coded conditions and unaware of the chemical names.

6-10. % hedgehog cells

29. The % hedgehog cells were determined based on the visual scoring of 100 cells per sample. No statistical analysis was applied to % hedgehog.

7. Results

30. In this section only outlines of the study results are described. The details are given in the study reports for each laboratory and the statistical analysis of results by Dr. Takashi Omori.

7-1.Control groups

31. Figs. 1 and 2 summarize the lab-orderly means of % DNA in tail (Estimate) in the vehicle and positive control EMS groups in the liver and the stomach, respectively.

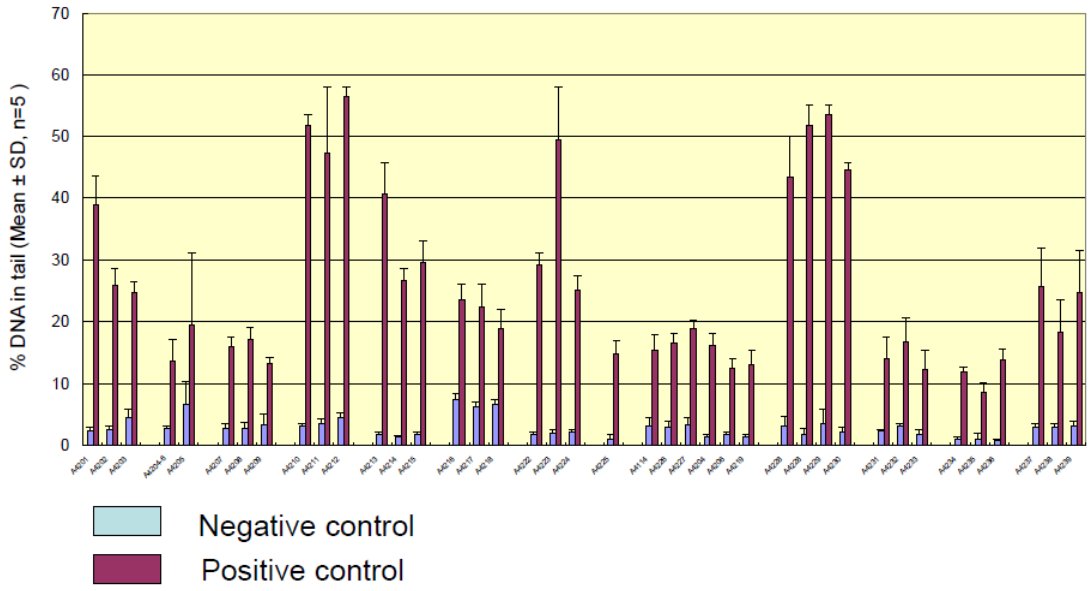


Fig. 1 Estimate (% DNA in tail) in the liver of vehicle control and positive control EMS groups in each lab. Each column shows mean ± S.D. (n=5 animals).

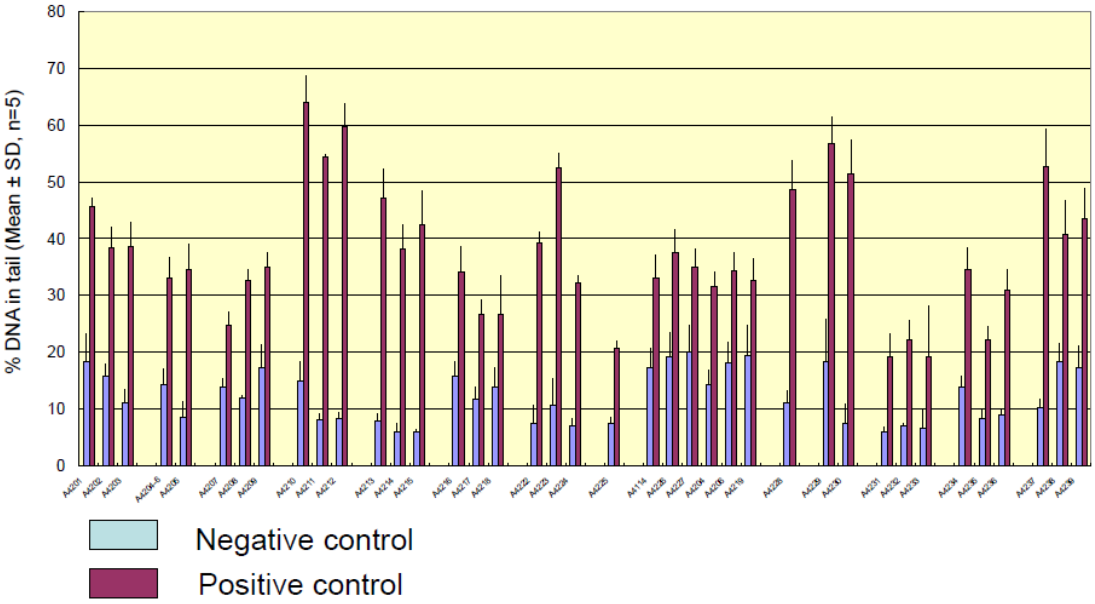


Fig. 2 Estimate (% DNA in tail) in the stomach of vehicle control and positive control EMS groups in each lab. Each column shows mean ± S.D. (n=5 animals).

7-1-1.Vehicle control groups

32. Figs. 3 and 4 are enlarged-figures of the lab-orderly Estimate in the vehicle control groups in the liver and the stomach, respectively. All the values in the stomach satisfied the preferred data-acceptance criteria of 1-20%, and there was little within/between-laboratory variability. The values for the liver also met the data-acceptance criteria of 1-8% except for one experiment

with a coded test chemical, trisodium EDTA monohydrate in Lab I, where the actual mean control value in the liver was below the lower limit of the recommended range at 0.7%. This was not considered to be a serious deviation. In the liver, there was some between-laboratory variability, but within-laboratory variability was minimal, indicating that experiments were well-controlled in each laboratory. Figs. 5 and 6 show the vehicle-orderly Estimate in the vehicle control groups (i.e. data grouped according to the different vehicles used) in the liver and the stomach, respectively. In both organs, the control values were generally slightly higher with corn oil compared with the other aqueous vehicles.

33. As a deviation from the validation study protocol v.14.2, in Lab M DNA was stained with ethidium bromide (EtBr) instead of with SYBR Gold, since this laboratory routinely uses EtBr. Thus the control data were separately shown in Fig. 9. Since EtBr is widely used for staining DNA in the Comet assay, the VMT decided to accept the data from Lab M, although this deviation should be taken into consideration for the data review. The negative control values in both organs therefore satisfied the data acceptance criteria.

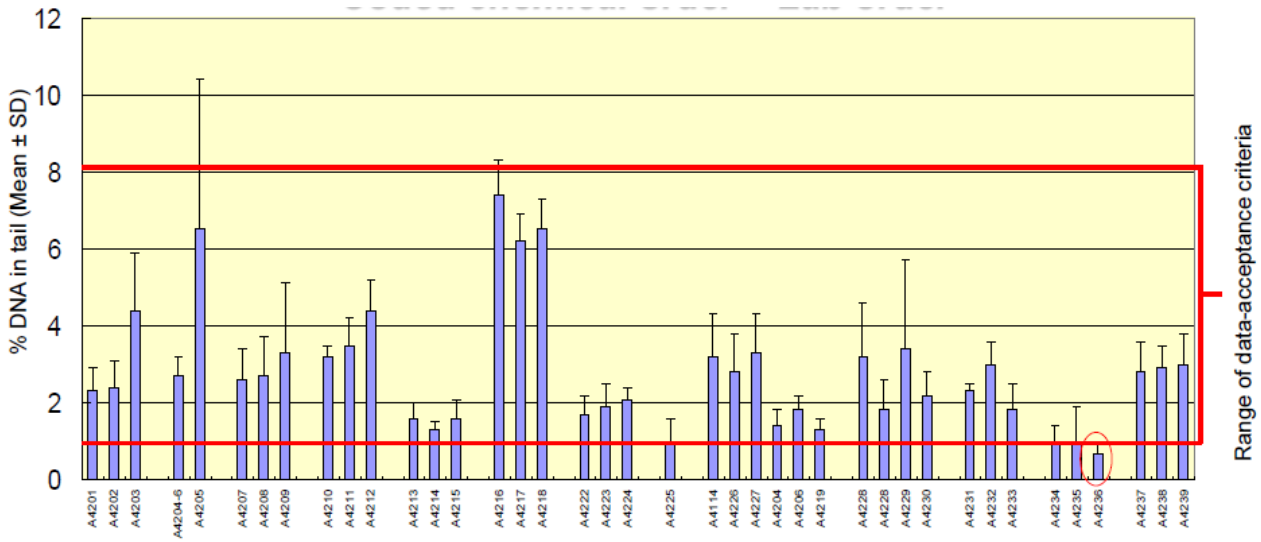


Fig. 3 An enlarged figure of Estimate (% DNA in tail) in the liver of vehicle control group in each lab. Each column shows mean ± S.D. (n=5 animals). All values satisfied the data acceptance criteria 1-8% except A4236.

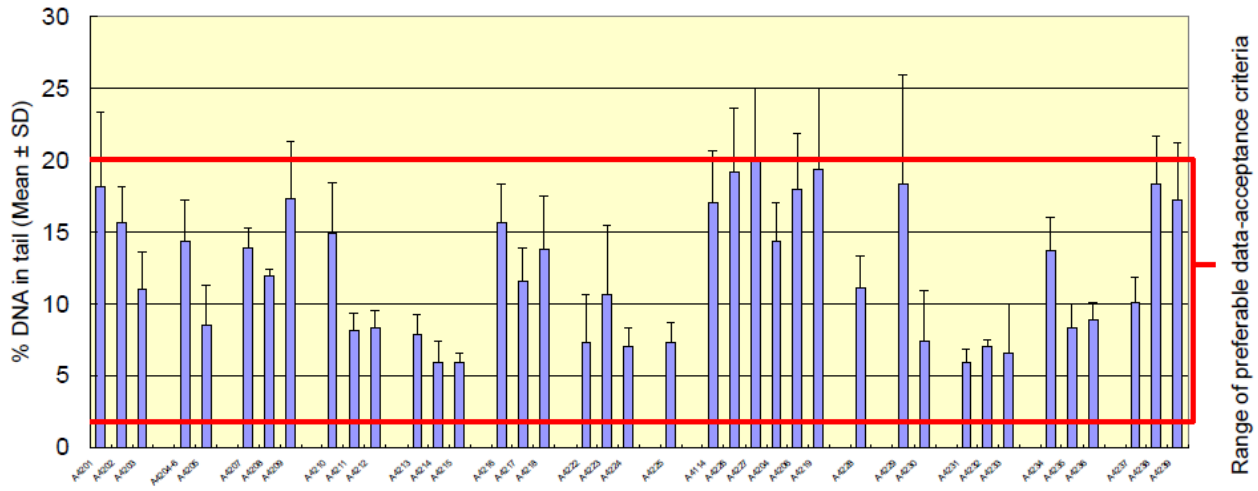


Fig. 4 An enlarged figure of Estimate (% DNA in tail) in the stomach of a vehicle control group in each lab. Each column shows mean ± S.D. (n=5 animals). All values satisfied the preferable data acceptance criteria 1-20%.

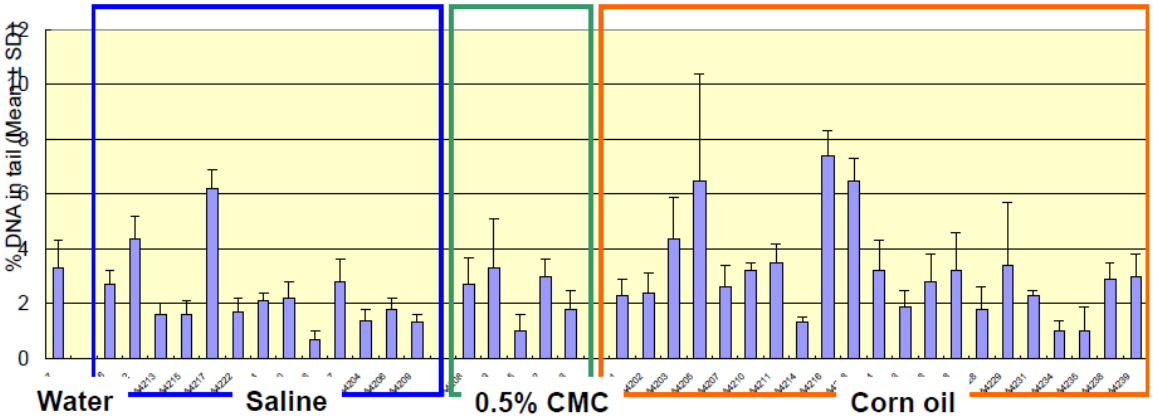


Fig. 5 An enlarged figure of Estimate (% DNA in tail) in the liver of each vehicle control group. Each column shows mean ± S.D. (n=5 animals). Mean ± S.D.; Saline: 2.6 ± 1.7 (n=9), 0.5% CMC: 2.1 ± 0.8 (n=5), Corn oil: 3.1 ± 1.7 (n=16)

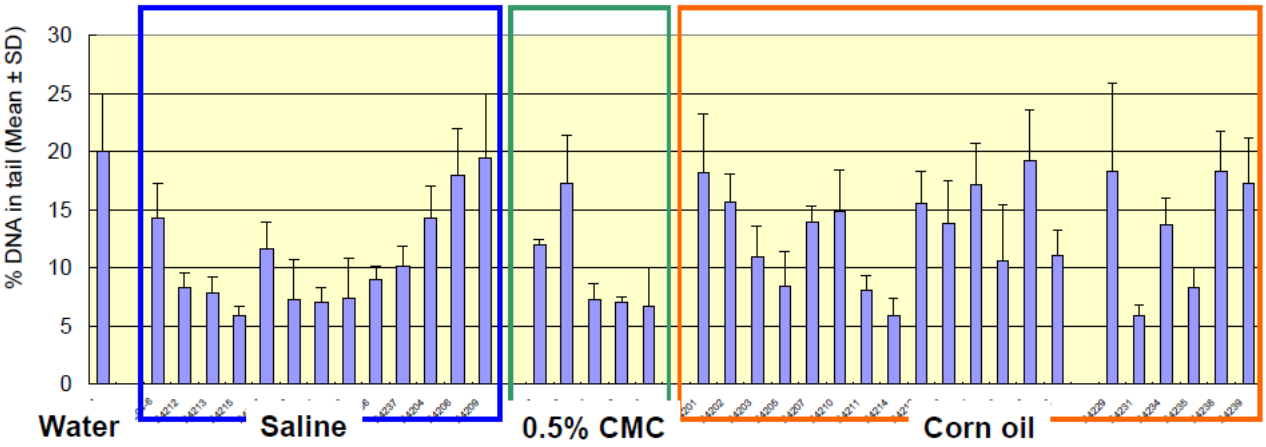


Fig. 6 An enlarged figure of Estimate (% DNA in tail) in the stomach of each vehicle control group. Each column shows mean ± S.D. (n=5 animals).Mean ± S.D.; Saline: 9.0 ± 2.6 (n=9), 0.5% CMC: 8.7 ± 2.4 (n=5), Corn oil: 14.0 ± 4.5 (n=16)

7-1-2.Positive control groups

34. Figs. 7 and 8 show Effect (diff.) of mean %DNA in tail values between the vehicle control group and the positive control group in the liver and the stomach, respectively. All Effect (diff.) values were statistically significant using Student"s t-test (one-sided, $p<0.025$), both in the liver and the stomach, and all had 5% or higher % DNA in tail values. Therefore, it was judged that all the positive control values satisfied the primary data-acceptance criteria. Regarding the magnitude of increased % tail DNA in both organs, there was some between-laboratory variability, but within-laboratory variability was minimal (see error bars on data shown in Figs. 1 and 2), indicating that experiments were well controlled in each laboratory.

35. As mentioned in the section 7-1-1., in Lab M the DNA was stained with EtBr instead of with SYBR Gold, and thus the control data are separately shown in Fig. 9. The negative control values and positive control responses in both organs satisfied the data acceptance criteria.

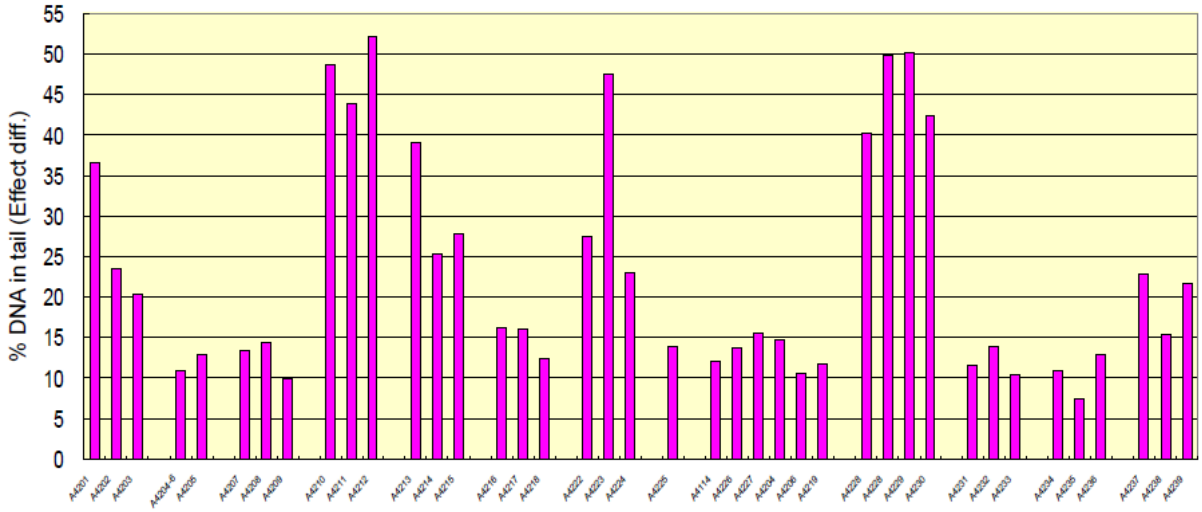


Fig. 7 Effect (difference between means of negative and positive control groups) of % DNA in tail in the liver in each lab. All data show statistically significant increases in Student"s t-test (one-sided, $p<0.025$) and 5% or higher values, satisfying the primary data acceptance criteria.

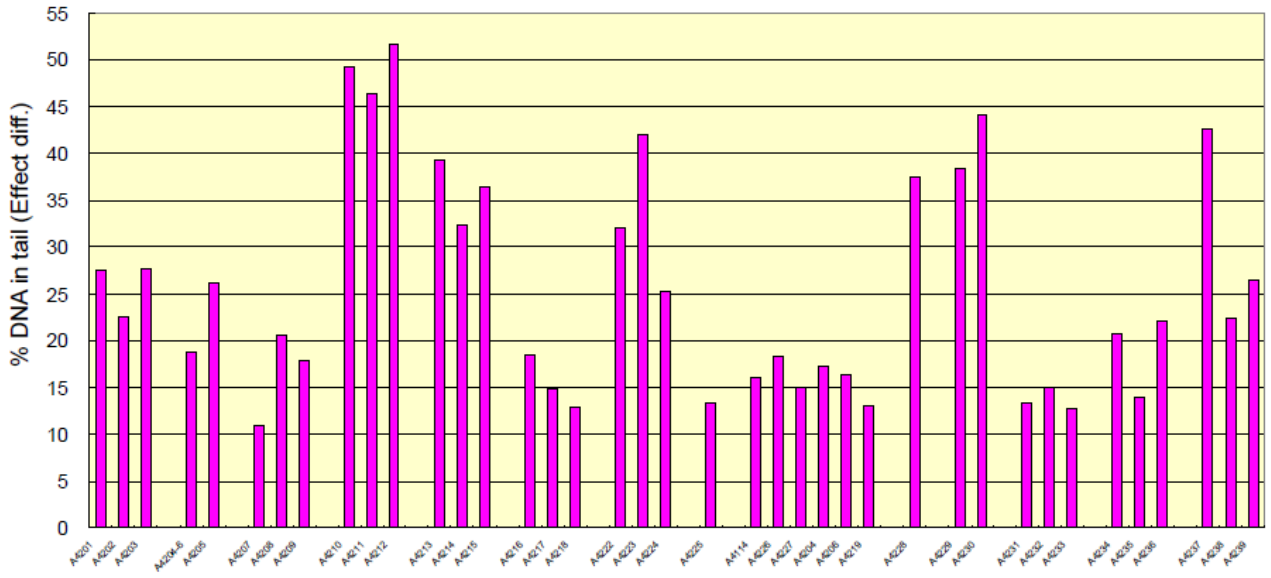


Fig. 8 Effect (difference between means of negative and positive control groups) of % DNA in tail in the stomach in each lab. All data show statistically significant increases in Student"s t-test (one-sided, $p<0.025$) and 5% or higher values, satisfying the primary data acceptance criteria.

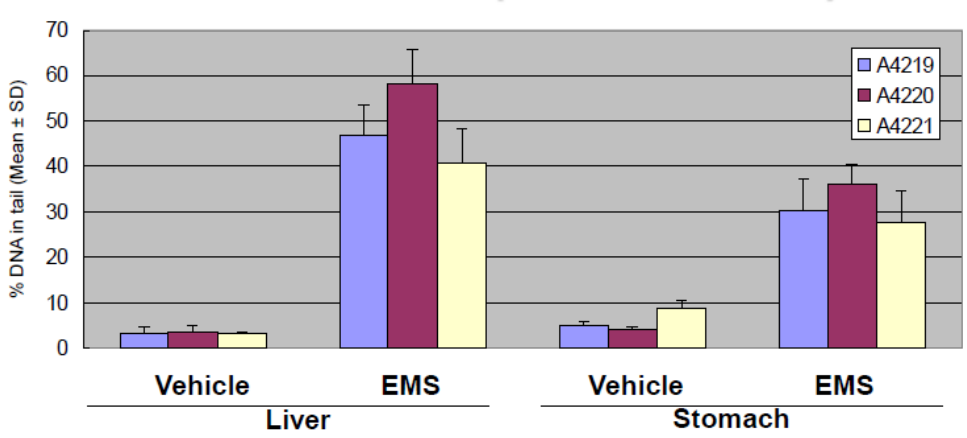


Fig. 9 Estimate (% DNA in tail) in the liver and stomach of vehicle control and positive control EMS groups at Lab M. Each column shows mean ± S.D. (n=5 animals).

As cells were stained with ethidium bromide, the data are shown separately.

7-2. Acceptability of data for test chemical evaluation

36. Some deviations to protocol 14.2 were noted as mentioned in the section 7-1. Although no reports have been found directly comparing the sensitivity of detection of DNA damage with SYBR Gold and EtBr, it has been reported that there was no significant difference in the sensitivity between SYBR Gold and propidium iodide (PI), although SYBR Gold was more convenient for image analysis purpose (Kirkland, et al, 2008b). When considering that EtBr and PI are equally used to stain DNA, it is speculated that SYBR Gold and EtBr would be as

comparable as SYBR Gold and PI. Thus the VMT considered that the deviations in the use of EtBr were noncritical and did not affect the evaluation of coded test chemicals with the Comet assay.

37. At a meeting at Huntingdon held on February 7-9, 2011, three other deviations from the validation study protocol were pointed out as shown in the following items 1) to 3). In addition, at the Kyoto meeting held on September 12-13, 2011, an issue relating to histopathological analysis was noted, and is shown below as item 4). After those meetings, all the issues were resolved as detailed in each item below:

1) Test chemical ampicillin trihydrate (Lab L) needed to be retested because no toxicity was observed at the highest dose level of 100 mg/kg. This issue occurred because of a misunderstanding related to the validation study protocol, i.e., ampicillin trihydrate was soluble up to 10 mg/mL in saline which was the first choice of vehicle in the validation study protocol, and Lab L believed that the solubility limit in saline was an appropriate criterion by which to select the highest dose level (since dosing volume was designated as 10 mL/kg, the highest dose level was set at 100 mg/kg). When it was pointed out that higher doses could be achieved by using corn oil, Lab L accepted that higher doses could be tested, and reexamined ampicillin trihydrate up to 2000 mg/kg (upper limit dose of the validation study protocol) with corn oil as a vehicle.
2) It appeared that test chemical hydroquinone (Lab N) needed to be retested, because no toxicity was observed in the highest dose level of 500 mg/kg. In accordance with the request, Lab N retested hydroquinone with a top dose level of 750 mg/kg. However, 2 out of 5 animal deaths were observed at this dose level, indicating that hydroquinone exhibited a steep toxicity curve, and induced severe toxicity with only a slight increase in the dose. Therefore, 500 mg/kg of hydroquinone was considered to be the maximum tolerated dose, and the Comet assay data up to 500 mg/kg were considered acceptable for the evaluation.
3) Test chemical sodium arsenite needed to be retested because an equivocal judgment was reached following statistical analysis conducted by the VMT, whereas Lab M judged sodium arsenite as positive based on their overall analysis. Since Lab M did not accept the need for a retest, Lab O reexamined sodium arsenite.
4) Test chemicals *N*-nitrosodimethylamine and 1,2-dimethylhydrazine (both tested in Lab L) induced increased % DNA in tail in the liver at all dose levels, but histopathology for the liver was only examined at the highest dose levels, and cytotoxic changes were observed. In those cases, histopathology data were also needed for the mid and low dose groups. The VMT therefore asked for a retest with additional histopathology, but Lab L was unable to do this and so Lab O retested *N*-nitrosodimethylamine and 1,2-dimethylhydrazine on their behalf.

38. From the viewpoint of data-acceptance criteria (as defined above), the VMT considered that all the data on coded test chemicals, including results of retests, would be acceptable for the

evaluation of the predictive capability of the *in vivo* Comet assay.

7-2. Coded test chemical groups

39. Table 2 shows the summary of results on all coded test chemicals. The table includes three types of judgment on the Comet assay results, i.e., laboratory judgment, statistical judgment, and final judgment. These are described as follows:

- Laboratory judgment is the conclusion of each testing facility, either "Positive", "Negative" or "Equivocal" based on their own statistical analysis and histopathological consideration. Some laboratories also considered their own historical control range.
- The statistical judgment was simply based on the statistical analysis without consideration of histopathology or historical control range, and it is shown as "Increase", "Decrease", "Equivocal", or "No change" in % DNA in tail. "Increase" means a statistically significant increase in both Dunnett's and linear Trend tests, "Decrease" means a statistically significant decrease in both statistical tests, "Equivocal" means a statistically increase or decrease in either statistical test but not in both, and "No change" means no significant change according to both statistical tests.

40. The final judgment was decided through discussion with all participants at the Kyoto meeting, and was based on the consideration of both statistical analysis and histopathology findings. Chemicals were judged to be "Positive", "Negative" or "Equivocal". When a relevant increase in % DNA in tail was noted in either the liver or the stomach, it was simply judged to be "Positive".

Table 2. Summary of 4th Phase-2nd Step Validation Study Results

Genotoxic carcinogens

Chemical (CASRN)	Vehicle	Dose mg/kg	Lab judge	Stat judge	Final judge	Note	
						In vivo genotoxicity	Other information
2-Acetylaminofluorene (53-96-3)	Corn oil	250, 500, 1000	Negative	No change	Negative	UDS, MN, Comet, TG (L): +	Ames, CA: + Rat Carc.: L, Mgl, Ski
Acrylonitrile (107-13-1)	Corn oil	15.7, 31.3, 62.5	Negative (within historical control range)	Equivocal (Trend: L), Decrease (S)	Positive (L)	UDS, MN, TG: - Comet: +	Ames, CA: + Rat Carc.: Zy, Nrv, Orc. Smi, S, Mgl, Nas
		15.7, 31.3, 62.5	Negative (within historical control range)	Increase (L)			
o-Anisidine (90-04-0)	Corn oil	150, 300, 600	Negative	No change	Negative	UDS, MN: - Comet, TG (Ubl): +	Ames, CA: + Rat Carc.: Kid, Thy, Ubl
Azidothymidine (30516-87-1)	0.5% CMC	500, 1000, 2000	Positive	Increase(L), Equivocal (Trend:S)	Positive (L)	MN: +	Ames: -, CA: + Rat Carc.: Vag
Benzene (71-43-2)	Corn oil	500, 1000, 2000	Negative	No change	Negative	MN, Comet: + TG (L): -	Ames: -, CA: + Rat Carc.: Zy, Nas, Orc, Ski, S, Vsc
Busulfan (55-98-1)	Corn oil	10, 20, 40	Negative	No change	Negative	MN, Comet: +	Ames, CA: + Mouse Carc.: Hmo, Ova
Cadmium chloride (10108-64-2)	Saline	20, 40, 80	Positive	Increase(L), Equivocal (Du: S)	Positive (L)	MN: + or - Comet: -	Ames: -, CA: + Rat Carc.: Hmo, Kid, Lun, Pro, Tes

Table 2. (continued)

Genotoxic carcinogens (continued)

Chemical (CASRN)	Vehicle	Dose mg/kg	Lab judge	Stat judge	Final judge	Note	
						In vivo genotoxicity	Other information
p-Chloroaniline (106-47-8)	Corn oil	37.5, 75, 150	Positive	Increase (L, S)	Positive (L, S)	MN: equivocal Comet: +	Ames: +, CA: + or - Rat Carc.: Spl
Cisplatin (15663-27-1)	0.5% CMC	6, 12.5, 25	Positive	Increase (L)	Positive (L)	MN, Comet, TG (L): +	Ames, CA: +
2,4-Diaminotoluene (95-80-7)	Saline	37.5, 75, 150	Equivocal	Increase (L)	Positive (L)	UDS, TG (L): + MN, Comet: + or -	Ames, CA: + Rat Carc.: L, Mgl
		100, 150, 200	Positive	Increase(L)			
1,2-Dibromoethane (106-93-4))	Corn oil	25, 50, 100	Positive	Increase (L, S)	Positive (L, S)	UDS, Comet: + MN, TG (L): -	Ames, CA: + Rat Carc.: Nas, Per, Pit, S, Vsc, L, Lun, Mgl
1,3-Dichloropropene (542-75-6)	Corn oil	50, 100, 200	Positive	Increase (L)	Positive (L)	UDS, MN: - Comet: +	Ames, CA: + Rat Carc.: L
1,2-Dimethylhydrazine 2HCl (306-37-6)	Saline	6.25, 12.5, 25	Positive	Increase (L)	Positive (L)	UDS, MN, Comet: +	Ames, CA: + Rat Carc: not available Mouse Carc.: Lung, Vsc
		1.56, 3.13, 6.25	Positive	Increase (L)			
Hydroquinone (123-31-9)	Saline	125, 250, 500	Negative	No change	Negative	MN: + (aneugen)	Ames: -, CA: + Rat Carc.: Kid, Hmo
Methyl methanesulfonate (66-27-3)	Saline	20, 40, 80	Positive	Increase (L, S)	Positive (L, S)	UDS, MN, Comet: + TG: + or -	Ames, CA: + Mouse Carc.: Hmo, Lun
N-Nitrosodimethylamine (62-75-9)	Saline	2.5, 5, 10	Positive	Increase (L)	Positive (L)	UDS, MN, Comet: +	Ames, CA: + Rat Carc.: L, Kid, Lun, Tes, Vsc
		0.63, 1.25, 2.5	Positive	Increase (L)			

Table 2. (continued)

Genotoxic carcinogens (continued)

Chemical (CASRN)	Vehicle	Dose mg/kg	Lab judge	Stat judge	Final judge	Note	
						In vivo genotoxicity	Other information
4,4'-Oxydianiline (101-80-4)	0.5% CMC	50, 100, 200	Negative	Decrease (S)	Negative	UDS: - MN, Comet: +	Ames, CA: + Rat Carc.: L, Thy
Sodium arsenite (7784-46-5)	Saline	7.5, 15, 30	Positive	Equivocal (Trend: L)	Equivocal	MN: +	Ames: -, CA: + Rat Carc.: not available Mouse Carc: L
		7.5, 15, 30	Negative	Equivocal (Dunnett: L)			
Thioacetamide (62-55-5)	Saline	19, 38, 75	Negative (due to toxicity)	Increase (L, S)	Positive (S)	MN, Comet: +	Ames, CA: - Rat Carc.: L, Hepatotoxicant

Genotoxic non-carcinogens

Chemical (CASRN)	Vehicle	Dose mg/kg	Lab judge	Stat judge	Final judge	Note	
						In vivo genotoxicity	Other information
9-Aminoacridine hydrochloride monohydrate (52417-22-8)	Corn oil	15.7, 31.3, 62.5	Negative	No change	Negative	No data	Ames: +
p-Anisidine (104-94-9)	0.5% CMC	125, 250, 500	Negative	No change	Negative	No data	Ames, CA: +
2,6-Diaminotoluene (823-40-5)	Corn oil	150, 300, 600	Positive	Increase (L)	Positive (L)	UDS, MN, Comet: + or -	Ames, CA: +
5-Fluorouracil (51-21-8)	Saline	25, 50, 100	Negative	No change	Negative	MN: + , Comet: -	Ames, CA: -

8-Hydroxyquinoline (148-24-3)	Corn oil	125, 250, 500	Negative	Decrease (S)	Negative	UDS, MN, Comet: -	Ames, CA: +
p-Phenylenediamine dihydrochloride (624-18-0)	Saline	25, 50, 100	Negative	No change	Negative	MN, Comet: -	Ames, CA: +

Table 2. (continued)

Non-genotoxic carcinogens

Chemical (CASRN)	Vehicle	Dose mg/kg	Lab judge	Stat judge	Final judge	Note	
						In vivo genotoxicity	Other information
Chloroform (67-66-3)	Corn oil	125, 250, 500	Negative (due to toxicity)	Increase (L)	Negative	UDS, Comet, TG (L): - MN: + or -	Ames, CA: - Rat Carc.: Kid, L Hepatotoxicant
Diethanolamine (111-42-2)	Saline	175, 350, 700	Negative	No change	Negative	MN: -	Ames, CA: - Rat Carc: - Mouse Carc. (dermal): L
Di(2-ethylhexyl)phthalate (117-81-7)	Corn oil	500, 1000, 2000	Negative	No change	Negative	UDS, MN, Comet, TG: -	Ames, CA: - Rat Carc.: L Peroxisome proliferator
Ethanol (64-17-5)	Saline	500, 1000, 2000	Negative	No change	Negative	MN: -	Ames, CA: - Rat Carc.: Adr, L, Pan, Pit Hepatotoxicant
Methyl carbamate (598-55-0)	Saline	500, 1000, 2000	Negative	No change	Negative	MN: -	Ames, CA: - Rat Carc.: L Hepatotoxicant
Saccharin (81-07-2)	Corn oil	500, 1000, 2000	Negative	No change	Negative	MN, TG (L): - Comet: +	Ames, CA: - Rat Carc.: Ubl
o-Phenylphenol sodium salt (132-27-4)	Corn oil	250, 500, 1000	Negative	No change	Negative	MN, CA: - Comet: + or -	Ames: - CA: + or - Rat Carc.: Kid, Ubl

Lab judge: Judgment of each lab; Stat judge: Judgment based on statistical analysis results. 'Increase' or 'Decrease' means statistically significant changes of % DNA in tail in both Dunnett's (Du) and linear Trend (Trend) tests. 'Equivocal' means the statistically significant changes in either of statistics; Final judge: Final judgment in consideration of histopathology; UDS: Unscheduled DNA synthesis assay with rat liver or stomach; MN: Micronucleus assay with mice and/or rats; TG: Gene mutation assay with transgenic mice and/or rats; CA: Chromosome aberration assay with mammalian cells or rodents;
L: Liver; S: Stomach; Adr: Adrenal gland; Hmo: Hematopoietic system; Kid: Kidney; Lun: Lung; Mgl: Mammary gland; Nas: Nasal cavity; Nrv: Nervous system; Orc: Oral cavity; Ova: Ovary; Pan: Pancreas; Per: Peritoneal cavity; Pit: Pituitary gland; Pro: Prostate; Ski: Skin; Smi: Small intestine; Spl: Spleen; Tes: Testes; Thy: Thyroid gland; Ubl: Urinary bladder; Vag: Vagina; Vsc: Vascular system; Zy: Zymbal's gland

Table 2. (continued)

Non-genotoxic non-carcinogens

Chemical (CASRN)	Vehicle	Dose mg/kg	Lab judge	Stat judge	Final judge	Note	
						In vivo genotoxicity	Other information
Ampicillin trihydrate (7177-48-2)	Saline	25, 50, 100	Negative	Equivocal (Trend:L)	Negative	MN: -	Ames: -, CA: +
	Corn oil	500, 1000, 2000	Negative	No change			
o-Anthranilic acid (118-92-3)	0.5% CMC	500, 1000, 2000	Negative	No change	Negative	MN: -	Ames: - CA: +
t-Butylhydroquinone (1948-33-0)	Corn oil	131.3, 262.5, 525	Negative (within historical control range)	Increase (L), Equivocal (Du: S)	Positive (L)	MN: -	Ames: - CA: +
Ethionamide (536-33-4)	Corn oil	125, 250, 500	Negative	Decrease (S)	Negative	No data	Ames: - CA: +
Isobutyraldehyde (78-84-2)	Corn oil	500, 1000, 2000	Negative	No change	Negative	MN: -	Ames: - CA: +
D,L-Menthol (15356-70-4)	Corn oil	500, 1000, 2000	Negative	No change	Negative	MN, Comet: -	Ames: - CA: +

Sodium chloride (7647-14-5)	Water	500, 1000, 2000	Negative	Decrease (S)	Negative	CA: - UDS (S): -	Ames, CA: - Gastrotoxicant
Trisodium EDTA monohydrate (10378-22-0)	Saline	500, 1000, 2000	Negative	No change	Negative	Comet: -	Ames, CA: -

7-3-1.Genotoxic carcinogens

41. Twelve of 19 genotoxic carcinogens tested induced a statistically significant increase % tail DNA in the liver and/or the stomach, and the statistical judgment for the 12 chemicals was "Increase". One of the carcinogens, cisplatin, gave positive comet responses in both stomach and liver despite being a cross-linking agent. In 10 of the 12 chemicals, these final "Positive" judgments were coincident with the laboratory's own judgment. However, for two of the chemicals, acrylonitrile and thioacetamide, the laboratory judgment was negative whilst the final judgment was positive.

42. Regarding acrylonitrile, in the first experiment it showed an equivocal response in the liver (a statistically significant increase in linear Trend test, but not in Dunnett's test) and a decrease in the stomach (a statistically significant decrease in both statistics). In the retest for the liver (not done for the stomach), it showed a statistically significant increase with both linear Trend test and Dunnett's test in the liver. Based on the statistics, the responses were concluded in the statistical judgements as "Increase" in the liver, and "Decrease" in the stomach. Acrylonitrile, however, was judged "Negative" in the testing facility, because the increased % tail DNA in the liver was within the historical control range of the testing facility (note: the decreased % tail DNA in the stomach was not discussed). No histopathological changes related to the chemical treatment were found in the liver. In the glandular stomach, oedema in the lamina propria/submucosal layer was found at 31.3 and 62.5 mg/kg/day (grade: very slight or slight). In the forestomach examined at 62.5 mg/kg/day, degeneration/necrosis in squamous cells (grade: moderate), oedema in the lamina propria/submucosal layer (grade: moderate), degeneration (vacuolar) in muscle fiber of the muscular layer (grade: slight), and ulcers (non-graded) were found. Therefore, for the final judgment, acrylonitrile was judged to be "Positive" in the liver in consideration of the reproducible results in two independent experiments and no cytotoxic finding in the liver. However, based on other published *in vivo* genotoxicity data with acrylotnitrile, where positive micronucleus and Comet assay responses have only been reported following iv or ip dosing in rats (see Appendix 3), it could be expected that it would not induce genotoxic effects in liver or stomach following oral dosing. Therefore, the laboratory's own conclusion of "negative" may be seen as consistent with the expected responses following oral dosing in the rat.

43. Thioacetamide induced a statistically significant increase in % tail DNA in the liver and stomach, and the statistical judgment was "Increase" in both organs. However, it was judged "Negative" in the testing facility, because cytotoxicity was noted in both organs following histopathological examination and/or increased % hedgehogs (liver). In the histopathology of the liver, hepatocellular degeneration (grade: minimal to moderate), central vein phlebitis (minimal to moderately severe), increased mitosis (minimal or slight), and bile duct hyperplasia (minimal) were found in all treatment groups. In the stomach, no abnormality was noted at 19 or 38 mg/kg/day, but erosion/ulcer (one of 6 rats, no-graded), and forestomach gastritis (one of 5

rats, no-graded) were found at 75 mg/kg/day. The VMT and participants in the Kyoto meeting considered that clear histopathological changes indicating cytotoxicity were observed in the liver, but not in the stomach. Therefore, this chemical was considered to be "Positive" in the stomach but negative in the liver due to cytotoxicity as the final judgments. Thioacetamide is a known (hepato)toxic agent. Dietary administration of thioacetamide caused liver cancer (hepatocellular carcinoma) in mice and rats and tumors of the bile duct in rats. Thioacetamide is oxidized to thioacetamide S-oxide which is not the reactive metabolite responsible for its toxicity. Thioacetamide S-oxide is further metabolized to a reactive intermediate which can either bind to liver liver macromolecules or be further degraded to acetamide and polar products. Thioacetamide is metabolized *in vivo* to acetamide which is itself carcinogenic (IARC, 1987; HSDB, 2003). Thioacetamide was negative in Ames and *in vitro* CA tests. Given that thioacetamide has been reported to induce micronuclei in mouse bone marrow following oral dosing, and induced comets in mouse stomach, colon and bladder following ip dosing, it may be expected that it would induce comets in rat stomach following oral dosing, and therefore the final judgment call of "Positive" may be consistent with expectations. The lack of published data on genotoxic effects in the liver following oral dosing, but lack of comet induction in the liver following ip dosing suggest that induction of DNA damage in rat liver following oral dosing may not be expected, and therefore the final judgment that the increased % tail DNA in the liver of rats was due to cytotoxicity rather than genotoxicity in this study may be consistent with this expectation.

44. For the remaining 10 genotoxic carcinogens, the laboratory and final judgements were in agreement. The expected and actual responses in liver and stomach can be summarised as follows:

45. Azidothymidine (AZT) was positive in the in vitro CA test, and it was also reported positive in Ames strain TA102 in the absence of S9 (IARC, 2000). After re-evaluation it was considered equivocal in the mouse lymphoma assay in the presence of S9 but negative in the absence of S9 by Seifried et al (2006), but reported positive in the absence of S9 by Wang et al (2009). It was also positive for *hprt* and *tk* mutations in TK6 cells in the absence of S9 (Meng, et al 2000; Torres, et al, 2007). It was also positive for induction of MN in blood reticulocytes of mice in vivo after repeated oral dosing (NTP), and also induced MN in rats after oral administration (Ayers, et al, 1996; Vikram, et al, 2008). Administration of AZT by gavage induced vaginal squamous-cell carcinomas in mice, and a low incidence of these tumors in rats treated with the highest dose. AZT is incorporated into nuclear and mitochondrial DNA in mammalian cells. It appears to cause mutations primarily by inducing large deletions, consistent with its action as a DNA chain terminator (IARC, 2000). Based on the above analysis AZT would be expected to be positive for comets in both liver and stomach. It was in fact positive in liver but gave an equivocal response (there was a significant trend test) in stomach.

46. Cadmium chloride was not mutagenic to bacteria. It was positive for induction of CA and MN in vitro, and induced MN in rats after repeated intraperitoneal dosing (Celik, et al, 2005), although Mavournin, et al (1990) concluded it was negative for MN induction in mice after administration in drinking water for 1, 3 and 7 days. It gave negative results in a Comet assay with mice treated by ip injection at single dose level. Oral administration of cadmium chloride to rats increased the incidence of large granular lymphocytes, leukaemia, prostate tumors, and testis tumors in rats. When ingested, most of the cadmium passes through the gastrointestinal tract without being absorbed. When absorbed, cadmium will bind to metallothionein, forming a cadmium–metallothionein complex that is transferred (via blood) primarily to the liver and the kidney. The genotoxicity of cadmium has to be explained by indirect mechanisms. Frequently discussed mechanisms are related to oxidative stress, the inhibition of DNA-repair systems, effects on cell proliferation, and on tumour-suppressor functions (IARC, 2009). Based on the above analysis it is possible that cadmium chloride might induce comets by indirect mechanisms in both stomach and liver. It was positive in liver but gave an equivocal response in both duodenum and stomach.

47. *p*-Chloroaniline was positive in Ames and in vitro CA tests but appears to be dependent on metabolism for its full expression.. It induced MN in mice treated by oral administration for 3 days at a dose level in the range of the LD_{50}, but not after single dosing. It gave positive results in a Comet assay in various organs in mice. Oral administration of *p*-chloroaniline induced sarcomas of the spleen and splenic capsule in male rats (IARC, 1993). Whether the mechanism of carcinogenesis is mediated through genotoxic or non-genotoxic events is unresolved. *p*-Chloroaniline causes methaemoglobinaemia (IARC, 1993) and is slightly irritating to eyes (HSDB, 2005). Based on the above analysis *p*-chloroaniline would be expected to induce comets in stomach and liver when given orally by 2 administrations, and was, in fact, positive in both liver and stomach.

48. Cisplatin was positive in the Ames test in the absence and presence of S9, and positive in in vitro CA and *hprt* mutation tests in the absence of S9. It induced MN and CA in bone marrow of rats and mice after ip dosing, and induced DNA damage (comets) in colon, lung and brain of mice and mutation in liver in mice treated by ip injection. Cisplatin induced lung adenomas in mice and leukemia in rats by multiple intraperitoneal treatments (IARC, 1987). Cisplatin can react in a nonenzymatic manner with water in vivo to form monoaquo and diaquo species following dissociation of the chloride groups. These metabolites extensively bind to protein (>90%). Cisplatin can also react with DNA, forming both intrastrand and interstrand cross-links. DNA adducts formed by cisplatin inhibit DNA replication and transcription, and lead to breaks and miscoding (HSDB, 2005). The substance causes eye irritation or damage (HSDB, 2005). Based on the above, following oral dosing in an aqueous vehicle, cisplatin may be expected not to induce comets in stomach and liver. It was, in fact, positive for increased DNA

migration in the liver, but gave negative results in stomach.

49. 2,4-Diaminotoluene was mutagenic in the Ames test in the presence of S9, and induced CA, SCE and transformation of SHE cells in vitro. It was also positive in vitro in HepG2 cells for UDS, MN and comets (Severin et al, 2005). It was generally negative for induction of MN in vivo in mice and rats treated by ip or oral routes, but produced some weak positive effects at toxic doses in the rat. It was positive in a repeat dose liver MN study (Takasawa, et al, 2012). It was positive in a Comet assay in several organs including stomach, liver and kidney in mice (Sasaki, et al, 1999), but was negative in liver of rats after 28 days of oral dosing (Rothfuss et al 2010). In a mouse transgenic mutation assay, it induced mutation in liver after oral administration and weak effects in the kidney after topical application. It induced DNA adducts (Taningher, et al, 1995) and produced weak induction of UDS in Fischer-344 rat liver (George & Westmoreland, 1991). 2,4-Diaminotoluene produced hepatocellular carcinomas after oral administration (IARC, 1987). In several rodent species the major urinary metabolite was 2,4-diamino-5-hydroxytoluene; N-acetyl and glucuronide conjugates were also found. 2,4-Diaminotoluene is a mutagenic and hepatocarcinogenic aromatic amine, requiring metabolic activation. It binds to DNA covalently, and increases cell proliferation in livers of animals (HSDB, 2003). 2,4-Diaminotoluene is a skin and eye irritant (HSDB, 2003). Based on the above analysis, 2,4-diaminotoluene would be expected to induce comets in liver but not in stomach, and these in fact were the findings in this validation trial.

50. 1,2-Dibromomethane was mutagenic to bacteria, and it was clastogenic to mammalian cells in vitro. It was negative for MN in bone marrow and for transgenic mutations in liver of mice after ip treatment. However, it was positive for in a Comet assay in multiple organs including liver and stomach, and for induction of UDS in liver by ip or oral dosing. After oral administration, 1,2-dibromoethane induced squamous cell carcinomas of the forestomach in rats and mice, hepatocellular carcinomas in female rats, hemangiosarcomas in male rats, and alveolar/bronchiolar adenomas in mice (IARC, 1999). Human liver preparations metabolize 1,2-dibromoethane to water-soluble and irreversibly protein- and DNA-bound metabolites by both cytochrome p450 (CYP2E1 etc) and glutathione S-transferase enzymes. It binds covalently with DNA in vivo. 1,2-dibromoethane is considered to be a bifunctional alkylating agent which is capable of introducing cross links into biological materials (HSDB, 2005). It is an irritant to eyes, skin, and mucous membranes (HSDB, 2005). Based on the above analysis, 1,2-dibromomethane would be expected to induce comets in both stomach and liver when given orally, which was, in fact, the case in this validation trial.

51. 1,3-Dichloropropene (technical grade) was positive in the Ames test (± metabolic activation), but not in the *in vitro* chromosomal aberration (CA) test. It was negative for MN in bone marrow and in a liver UDS assay with male rodents treated by ip or oral dosing, but induced DNA

fragmentation (alkaline elution assay) in liver and gastric mucosa while not in lung, bone marrow and brain. However, 1,3-dichloropropene induced MN in the bone marrow of female mice after oral treatment, and DNA damage (comets) in multiple organs of mice after ip treatment. Its genotoxicity could in some cases be confounded by traces of one or more impurities, stabilizers or even its unknown composition. Oxidation of 1,3-dichloropropene leads to formation of the extremely mutagenic α-chloroacrolein. GSH depletion at higher doses seems to have a major influence on the genotoxicity findings in a number of *in vivo* studies since GSH conjugation is a major detoxification pathway for 1,3-dichloropropene. Technical-grade 1,3-dichloropropene (containing 1.0% epichlorohydrin), when given by gavage, produced tumors of the urinary bladder, lung and forestomach in mice and of the liver and forestomach in male rats (forestomach only in female rats). Inhalation exposure produced an increase in the incidence of bronchioalveolar adenomas in mice while no increase in tumors was seen in rats. After subcutaneous administration to mice, the cis-isomer produced malignant tumors at the site of injection. The principle metabolic pathway of 1,3-dichloropropene is conjugation with glutathione and elimination as mercapturic acids (IARC, 1999). In the trial 1,3-dichloropropene was positive in liver but negative in stomach, whereas based on the above analysis it might be expected to induce comets in both in stomach and liver.

52. 1,2-Dimethylhydrazine dihydrochloride has given conflicting evidence for genotoxicity in bacteria. No data are available on induction of micronuclei *in vitro*. A report on chromosome aberrations in CHL cells indicates a positive effect after a continuous treatment without metabolic activation (Ishidate, 1987); no concurrent cytotoxicity data are presented but common practice in those days was to aim for 50% reduction in confluence at the top concentration. 1,2-Dimethylhydrazine was positive *in vivo* in genotoxicity tests including UDS, MN or Comet in rats after oral treatment. 1,2-Dimethylhydrazine formed DNA adducts and induced gene mutations, DNA breaks and micronuclei *in vivo* in rodents. *In vitro*, it formed DNA adducts and induced UDS and gene mutations in mammalian cells (mutations at TK locus in L5178Y cells in absence of metabolic activation; Rogers & Back, 1981). After oral administration to rats, it was found to induce DNA damage in the liver Comet assay in both acute and repeated dosing regimens (Rothfuss, et al., 2010) and in multiple organs in rats and mice (Sasaki, et al., 2000). Whatever the route of administration, 1,2-dimethylhydrazine produced adenomas and adenocarcinomas of the colon, and to a lesser extent, of the small bowel in mice and rats. Tumors of the vascular system and liver have also been observed in mice and rats, respectively (CCRIS). 1,2-Dimethylhydrazine requires bioactivation to become mutagenic and alkylates DNA in several species *in vivo*. Dimethylhydrazine is metabolized by a sequence of oxidation steps, first dehydrogenation to azomethane, N-oxidation of this to azoxymethane and finally a C-oxidation to methylazoxymethanol. This last metabolite decomposes to give the highly reactive methyldiazonium ion (DNA alkylation) to which the carcinogenicity of the compound has been attributed (IARC, 1999). Contact with the substance may cause irritation to skin, eyes, and mucous membranes (HSDB, 2005). Based on the above 1,2-dimethylhydrazine would be expected to be

positive for comet induction in liver, but (despite the Sasaki et al, 2000 paper)would probably be expected to be negative in stomach.

53. Methyl methanesulfonate was positive in Ames and *in vitro* CA tests, and in many different kinds of *in vivo* genotoxicity tests including UDS, MN, Comet or TG mutation assays. Methyl methanesulfonate produced nasal tumors, tumors of the nervous system in rats treated by inhalation exposure and by subcutaneous administration. In mice, it increased the incidence of lung tumors and of lymphomas after oral administration (IARC 71, 1999). Methyl methanesulfonate is one of the monofunctional, methylating agents which produce primarily 7-methyl-guanine as an adduct (HSDB, 2008). Based on the above methyl methanesulfonate would be expected to induce comets in both liver and stomach, as was found to be the case in this validation trial.

54. *N*-nitrosodimethylamine was positive in Ames and *in vitro* CA/MN tests (mainly in presence of metabolic activation), and for many kinds of *in vivo* genotoxicity tests including UDS, liver Comet or TG mutation assay. Induction of MN *in vivo* is less convincing. *N*-Nitrosodimethylamine is an example compound for which a second *in vivo* assay (in addition to the MN assay) in a different tissue (typically liver) is necessary to detect its genotoxic potential. *N*-Nitrosodimethylamine produced liver tumors in male rats treated by sc injection. It induced tumors of nasal cavities and kidneys in rat after inhalation exposures. The metabolic activation of N-nitrosodimethylamine by CYP2E1 and following alkylation of DNA are important in its carcinogenic effect (IARC, 1987; HSDB, 2005). The liquid and vapor may be irritating to the skin or eyes (HSDB, 2005). Based on the above analysis, *N*-nitrosodimethylamine would be expected to induce comets in liver, but not in stomach, which in fact were the results obtained in this validation trial.

55. Six of the 19 genotoxic carcinogens tested failed to induce a statistically significant increase or decrease in % tail DNA in the liver and the stomach. These are discussed in detail below.

56. 2-acetylaminofluorene – 2-AAF is a well-known genotoxic carcinogen. Positive results are reported in almost all *in vivo* genotoxicity assays, i.e., rat liver UDS test, rat bone marrow micronucleus (MN) test, mouse Comet assay in the colon, liver, kidney and lung, and gene mutation assay in Big Blue mouse liver (note: reference papers on genotoxicity assay results are described in Appendix 3). It is reported that the oral administration to animals induced tumors in many organs such as the liver, urinary bladder, and kidney in many species (Nakajima, et al., 2012). To induce genotoxicity and carcinogenicity, metabolic activation of 2-AAF is required, and the critical activation pathway is known to convert 2-AAF to the *N*-hydroxy derivative and then to the *N*-SO_4 derivative (Gold LS.,2012).

57. In this validation study, this chemical was judged to be negative in both liver and stomach. 2-AAF was also examined in Phase 4-1 of the validation study, and a positive result (i.e., statistically significant increases in both Dunnett"s and Trend tests) in the liver was reported in only one of three laboratories testing this chemical. Therefore the overall judgment of 2-AAF would be considered negative in this validation study. It is relevant that 2-AAF reportedly failed to increase DNA migration in the rat liver when administered orally EPA, 1998). Interestingly, in the same report, it is mentioned that clear increased DNA migration was noted in the liver when 2-AAF was administered by i.p. injection, indicating that the route of administration may affect the genotoxic potential in the liver (US. EPA,1998). However, considering the overall genotoxic MoA of 2-AAF, it is unclear why 2-AAF failed to increase DNA migration in the liver under the conditions of this validation study and in other publications. When considering that the dose level usually used in the UDS assay is low (50 mg/kg or below) based on the reference article in OECD-TG486 (Rothfuss, et al., 2011), the lower dose levels may be a factor in understanding the negative results obtained at much higher doses in this validation study. The timing of tissue sampling may also play a role for the negative result because it is reported that a peak response in the rat liver UDS assay was at 12 hours after a single oral dose of 2-AAF at 50 mg/kg, although the positive findings were also noted at 2 and 24 hours after the treatment (Rothfuss, et al., 2011)). Another possible contributing factor is the formulation handling, e.g. sonication and/or heating of the dosing formulation. Clearly, further work is needed to understand why 2-AAF does not readily induce comets in rat liver after high oral doses.

58. *o*-Anisidine - This chemical is known to be oxidatively activated by peroxidase and cytochrome P-450 (CYP), and DNA adducts have been identified as deoxyguanosine adducts formed from a metabolite, *N*-(2-methoxyphenyl)hydroxylamine. In these investigations rats were treated i.p. with *o*-anisidine (0.15 mg/kg daily for 5 days) and DNA from several organs was analyzed by ^{32}P-postlabeling. Two *o*-anisidine-DNA adducts were detected in urinary bladder (4.1 adducts per 10^7 nucleotides), the target organ of carcinogenicity, and, to a lesser extent, in the liver, kidney and spleen (Madle, et al., 1994). In *in vivo* genotoxicity assays, this chemical is reported to be positive in the kidney, bladder, lung, stomach and colon in the rat Comet assay, and in the bladder in a gene mutation assay with Big Blue mice (see Appendix 3). In contrast, negative gene mutation results have been reported in the liver in the Big Blue mouse and the rat liver UDS test, and also in the mouse bone marrow MN test (see Appendix 3). Carcinogenicity was evaluated with *o*-anisidine hydrochloride by administering in feed to Fischer 344 rats at 5000 or 10000 ppm and B6C3F1 mice at 2500 or 5000 ppm for 103 weeks, then observed for 1 or 2 additional weeks. It was concluded that *o*-anisidine hydrochloride was carcinogenic for rats and mice, inducing transitional-cell carcinomas or papillomas of the bladder in both rats and mice and in both sexes of each species, transitional-cell carcinomas of the pelvis of the kidney in male rats, and follicular-cell tumors of the thyroid in male rats

(Stiborová, et al., 2005).

59. Based on the above reports, the primary target organ in carcinogenicity studies using *o*-anisidine hydrochloride is considered to be the urinary bladder. The expectation of similar target-organ specific genotoxic effects is supported by a series of data that DNA adducts are formed mainly in the bladder, and to a lesser extent in the liver, kidney, and spleen. Positive results were also obtained in the urinary bladder in the gene mutation assay using TG mice, and in the rat Comet assay (also positive in the kidney), whereas in contrast, negative results were obtained in the rat liver UDS assay, the mouse bone marrow MN assay, and the liver gene mutation assay using TG mice. Positive results in the lung, colon and stomach, which are non-target organs in carcinogenicity studies, are also reported in the rat Comet assay, but the increases in tail migration were noted in a limited testing condition, i.e., increased only at 8 hr after the single oral administration of 1000 mg/kg but not at 3 and 24 hrs. Dose-dependency was not examined, indicating that the positive findings in non-target organs might be considered questionable. Therefore, when considering the target organ specificity of this chemical, the negative judgment in this validation study may be considered consistent with the expectations. The negative result also indicates a known limitation of the standard protocol used in this validation trial with liver and stomach in that, other tissues may be the target organs for carcinogenicity. If target organ specific effects are anticipated or known, then those organs may be investigated using the Comet assay to help determine or investigate a potential genotoxic MoA in the carcinogenic target organ(s). Therefore, based on all the available data negative results might be expected in stomach and liver of rats after oral dosing of *o*-anisidine.

60. Benzene – Two-year carcinogenicity studies of benzene were conducted in F344/N rats and B6C3F1 mice. Doses of 0, 50, 100, or 200 mg/kg body weight benzene in corn oil (5 ml/kg) were administered by gavage to male rats, 5 days per week, for 103 weeks. Doses of 0, 25, 50, or 100 mg/kg benzene in corn oil were administered by gavage to female rats and to male and female mice for 103 weeks. There was clear evidence of carcinogenicity of benzene for both sexes in rats and mice. For male and female rats, benzene caused increased incidences of Zymbal's gland carcinomas, and squamous cell papillomas and squamous cell carcinomas of the oral cavity. In male rats, benzene also caused increased incidences of squamous cell papillomas and squamous cell carcinomas of the skin. For male and female mice, benzene caused increased incidences of malignant lymphomas, Zymbal's gland squamous cell carcinomas, alveolar/bronchiolar carcinomas and alveolar/bronchiolar adenomas or carcinomas (combined). In male mice, Harderian gland adenomas, and squamous cell carcinomas of the preputial gland were also increased. For female mice, benzene also caused increased incidences of ovarian granulosa cell tumors, ovarian benign mixed tumors, and carcinomas and carcinosarcomas of the mammary gland. Dose-related lymphocytopenia was observed for male and female F344/N rats and male and female B6C3F1 mice (NTP, 1978).

61. It is generally agreed that the toxicity of inhaled benzene results from its biotransformation to reactive species. Benzene is metabolized in the liver by cytochrome P-4502E1 (CYP2E1) to its major metabolites: phenol, hydroquinone, and catechol. The intermediate benzene oxide can also undergo ring opening to trans-trans muconic acid. Although there is a scientific consensus that metabolism of benzene is required for resultant toxicity and carcinogenic response, the role of a metabolite or metabolites of benzene in producing these adverse effects is controversial and more research data are needed to better define sequelae of pathogenesis. Current evidence indicates that benzene-induced myelotoxicity and genotoxicity result from a synergistic combination of phenol with hydroquinone, muconaldehyde, or catechol (NTP, 1986).

62. The bone marrow, Zymbal's gland, and Harderian gland all contain peroxidases, which can activate phenols to toxic quinones and free radicals. Sulfatases, which remove conjugated sulfate and thus reform free phenols, are also present at high levels in these target organs. The selective distribution of these two types of enzymes in the body may explain the accumulation of free phenol, hydroquinone, and catechol in the bone marrow and the resulting differences in target organ toxicity of benzene metabolites in humans and animals (NTP, 1986).

63. Molecular targets are considered to be mainly tubulin, topoisomerase II and histones, and DNA itself (oxidation and adduct formation) is considered less of a target. In genotoxicity studies, benzene exposure has been shown to induce aneuploidy in dividing cells, presumably through inhibition of tubulin assembly during mitosis. However, benzene exposure has failed consistently to induce point mutations in genotoxicity studies (NTP, 1986).

64. In *in vivo* genotoxicity assays, oral administration of benzene showed positive results in the rat bone marrow MN test, and the rat and mouse Comet assay in many organs including the liver and stomach. In contrast, inhalation of benzene failed to increase mutation frequency in the liver using the Big Blue mouse gene mutation assay (see Appendix 3).

65. In this validation study, benzene was judged to be negative in the liver and stomach, but this is not consistent with the previous report of the Comet assay (Sasaki , et al., 2000). However, considering the known genotoxic and carcinogenic MoA, the negative results found in this trial might be expected.

66. Busulfan – It is reported that this chemical is a direct-acting bi-functional alkylating agent that binds to cellular macromolecules including DNA, RNA, and proteins. Mono-adduct formation, intrastrand cross-links, and DNA-protein cross-links are reported. In rats, i.v. administration of busulfan for one year was reported to induce a variety of tumors in male rats, although the experiments could not be evaluated by IARC due to incomplete reporting. In mice, i.p. administration induced lymphomas in two studies, but did not increase the incidence of tumors in two other studies. Following i.v. administration to mice, increased incidences of thymic and ovarian tumors were reported (US EPA-IRIS, 2012).

67. The above report indicates that the major genotoxic MoA of busulfan is cross-link formation (US EPA-IRIS, 2012). When considering that simple mono-functional alkylating agents such as EMS or MMS clearly increased % tail DNA in this validation study, the "Negative" result of busulfan indicates that this chemical would be likely to induce DNA damage through cross-linking formation rather than mono-adduct formation *in vivo*. It is reported that cross-linking agents mitomycin C and cisplatin decreased % tail DNA compared with the negative control (IARC 100A, 2012, McKenna et al., 2003), and thus it can be expected that cross-linking agents might have induced decreases in % tail DNA in this validation study. However, the cross-linking agents busulfan and cisplatin, used in this validation study, failed to decrease % tail DNA, indicating that cross-linking agents could not be detected in *in vivo* Comet assay, at least under the conditions of this validation study. This may be due to low % tail DNA values in control tissues, particularly liver. Longer duration of electrophoresis might be needed to detect the decreased % tail DNA, since decreased DNA migration has been reported in kidney cells following longer duration of electrophoresis (McKenna, et al., 2003).

68. This chemical was optionally examined with the micronucleus assay using peripheral blood in Lab C and they reported that increased micronuclei were observed in all dose groups. This finding would be expected when considering the genotoxic MoA of busulfan.

69. Hydroquinone – The 2-year carcinogenicity study was conducted by administering 0, 25 or 50 mg/kg in deionized water by gavage to F344/N rats, 5 days/wk. Nearly all male rats and most female rats in all vehicle control and exposed groups had nephropathy, which was judged to be more severe in high-dose male rats. Hyperplasia of the renal pelvic transitional epithelium and renal cortical cysts were increased in male rats. Tubular cell hyperplasia of the kidney was seen in two high-dose male rats, and renal tubular adenomas were seen in low- and high-dose male rats. Mononuclear cell leukaemia in female rats occurred with increased incidences in the dosed groups.

70. B6C3F1 mice were given 0, 50 or 100 mg/kg on the same schedule as rats. Compound-related lesions observed in the liver of high-dose male mice included anisokaryosis, syncytial alteration and basophilic foci. The incidences of hepatocellular neoplasms, primarily adenomas, were increased in dosed female mice. Follicular cell hyperplasia of the thyroid gland was also increased in dosed mice (Nesslany, et al., 2007).

71. All renal tubule adenomas and all cases of renal tubule atypical hyperplasia occurred in areas of severe or end-stage chronic progressive nephropathy and the neoplasms were not otherwise confined to any particular part of the kidney. It is likely that the mode of carcinogenic action of hydroquinone is exacerbation of this natural disease process.

72. Hydroquinone is a metabolite of benzene. It is mutagenic *in vitro* and *in vivo*, having caused

genotoxicity or chromosomal aberrations in rodent bone-marrow cells, although the Ames test showed a negative result. At least a portion, if not all, of the chromosomal effects are caused by interference by hydroquinone or its metabolites with chromosomal segregation, probably due to interaction with mitotic spindle proteins (NTP, 1989). Although the dose routes used to demonstrate these effects in almost all of the studies *in vivo* were not p.o., but i.p. or s.c. injection, the major genotoxic MoA is considered to be interaction with mitotic spindle proteins (aneugenic effects). There were five genotoxicity studies by the oral route. These included a mouse bone-marrow cell MN test in which a weak, marginally positive response was obtained following a single oral dose of 80 mg/kg body weight. The remaining oral route studies showed no significant effect. They included a mouse bone-marrow cell MN test in which there was no genotoxic activity after exposure to a diet containing 0.8% hydroquinone for 6 days; two ^{32}P-post-labeling assays, one with targets of Zymbal‟s gland, liver, and spleen in SD rats, and the other with the kidney as target in F344 rats; and the last oral assay was for 8-hydroxydeoxyguanosine adducts in F344 rat kidney DNA. Thus, the evidence (and the database) for any genotoxic effect *in vivo* is sparse and none has been observed in the tumor target tissue, the kidney (NTP, 1989).

73. While glutathione conjugates could be responsible for the tumor induction, careful histology seems to show that the most actively toxic of several glutathione compounds tested, 2,3,5-triglutathion-S-yl hydroquinone, targets a very specific region of the kidney, the outer stripe of the outer medulla, whereas hydroquinone-associated adenomas are more randomly distributed and occur in the cortex as well as the medulla. A non-genotoxic MoA that involves exacerbation of a spontaneously occurring rodent renal disease, chronic progressive nephropathy (CPN), is proposed and evaluated. This disease is particularly prominent in male rats and the evidence is consistent with an absence of any human counterpart. Therefore, the increased incidence of renal tubule adenomas in hydroquinone-dosed male rats is without human consequence (NTP, 1989).

74. When considering the above genotoxic and carcinogenic MoA, the negative result in this validation study would be rational.

75. 4,4‟-oxydianiline - A bioassay of this chemical for possible carcinogenicity was conducted by feeding diets containing 200, 400, or 500 ppm of the test chemical to F344 rats and 150, 300, or 800 ppm to B6C3F1 mice for 104 weeks. 4,4'-Oxydianiline was carcinogenic for male and female F344 rats, inducing hepatocellular carcinomas or neoplastic nodules and follicular-cell adenomas or carcinomas of the thyroid. 4,4'-Oxydianiline was also carcinogenic for male and female B6C3F1 mice, including adenomas in the Harderian glands, hepatocellular adenomas or carcinomas in both sexes, and follicular-cell adenomas in the thyroid of females (McGregor, 2007).

76. This chemical is considered a mutagen due to the positive results in Ames and *in vitro*

chromosome aberration tests. Positive results in mice are also reported in the micronucleus test (i.p. injection) and the Comet assay (p.o.). However, a negative result is reported in the rat liver UDS test (p.o.). On the other hand, goitrogenic (anti-thyroid agent) effects of this chemical were also reported for rats and mice in the 90-day toxicity studies (McGregor, 2007). The authors indicate that rats or mice receiving anti-thyroid compounds may develop tumors of the thyroid gland. They also point out that the goitrogenic and carcinogenic effects of this chemical may be related to the structural similarity between the compound and thyroxin, and nuclear binding sites for thyroxin have been demonstrated in the rat liver and pituitary. In addition, 4,4'-oxydianiline was tested in a neonatal rat liver focus model, and showed clear evidence of hepatocarcinogenicity (NTP, 1980). Whilst the chemical did not show initiating activity in neonatal models, promoting activity, as indicated by increased number, size, or volume fraction of histochemically detected hepatic foci of cellular alteration, was evident for the chemical with previously demonstrated hepatocarcinogenicity.

77. In the current validation study, a statistically significant decrease in % tail DNA was found in the stomach, but negative results were reported in the liver. Since this chemical clearly has genotoxic potential, the negative results in UDS and Comet assays in rats, and the neonatal rat initiation assay model indicate that rats are less sensitive to the genotoxicity of this chemical when compared with mice. On the other hand, the previous carcinogenicity reports indicate that the carcinogenic MoA of this chemical might be related to the goitrogenic effect for the thyroid and the tumor promoting effect for the liver. Therefore, it can speculated that the carcinogenic activity of this chemical in rats might not involve a mutagenic mode of action. Further investigation of genotoxicity using rats would be needed to clarify the rat carcinogenic MoA, e.g., transgenic rat gene mutation assay or rat liver micronucleus assay.

78. In addition to the 6 negative results, sodium arsenite, induced increases in % tail DNA in the liver, but the statistical significance was only noted with the linear Trend test in Lab M, and with Dunnett‟s test in Lab O. This chemical was considered to produce a marginal increase in % tail DNA in the liver in two independent experiments, and the VMT finally judged this chemical “Equivocal”. Sodium arsenite was clastogenic to mammalian cells *in vitro*, but not mutagenic to bacteria. It induced MN and CA in the bone marrow of mice after intraperitoneal administration. Following long-term exposure through drinking water, increased DNA damage was observed in bone marrow and testicular cells of mice and in blood, liver, and kidney of rats by means of the Comet assay (Biswas, et al., 2007; Kadirvel, et al., 2007). In the latter study, also DNA-protein cross-links have been noted in all investigated tissues. Treatment with sodium arsenite once or four times induced a significant decrease in DNA migration in bladder and liver parenchymal cells of hepatic methyl donor sufficient (HMDS) mice, but in skin cells of HMDD mice (Tice et al., 1997). There is limited evidence in experimental animals for the carcinogenicity of sodium arsenite. It showed some evidence of renal tumor formation in female rats but not in males after oral administration (drinking water); tumour incidence did not reach significance.

Transplacental exposure via maternal oral exposure in mice (drinking water) to sodium arsenite during gestation induced lung, liver, ovary and adrenal tumors in the offspring in several studies, and the uterus in one study. Arsenicals do not react directly with DNA, but cells treated with low concentrations of trivalent arsenicals show increased oxidative DNA damage. As(III) acts as an aneugen by interfering with spindle function and causing micronuclei with centromeres, but at high (toxic) doses, it acts as a clastogen. Oxidative damage to DNA has been shown to cause changes in DNA methylation, suggesting a mechanism by which As(III) may induce this effect. Although not a mutagen, As(III) can enhance the mutagenicity of other agents by interference with DNA repair possibly through displacement of zinc at Zn fingers of proteins involved (IARC, 2009). It is irritating to the eyes (HSDB, 2003). Based on the above analysis, sodium arsenite would be expected to be negative for induction of comets in stomach and liver.

79. In % hedgehog analysis, only two "Positive" chemicals in the liver, 1,2-dimethylhydrazine dihydrochloride and *N*-nitrosodimethylamine, and one "Positive" chemical in the stomach, methyl methanesulfonate clearly increased hedgehogs in the positive organs with dose-dependency, but the other test chemicals including "Positive" chemicals were considered not to clearly affect % hedgehog.

7-3-2.Genotoxic non-carcinogens

80. One of six genotoxic non-carcinogens tested, 2,6-diaminotoluene, induced a statistically significant increase in % tail DNA in the liver, and the statistical judgment was "Increase" in the liver. In histopathology for the liver, no abnormality was noted at 150 mg/kg/day, and mitoses in hepatocytes and hemopoiesis were less frequent or absent at 300 and 600 mg/kg/day. Because of no clear cytotoxicity in the liver, the final judgment was "Positive", which was coincident with the lab judgment. No changes of % hedgehog were noted in all test chemicals in this category. 2,6-Diaminotoluene was positive in Ames and in vitro CA tests, but did not induce transformation in SHE cells. Results from in vivo genotoxicity studies were also conflicting. Relatively high doses of 2,6-diaminotoluene induced UDS, MN, and comets in rats after oral administration, and Rothfuss et al (2010) reported positive Comet results in rat liver after 28-day repeat oral dosing but was negative in an acute study at doses nearly 10-fold higher. Negative Comet results were reported in all organs examined following oral administration to mice (Sasaki et al, 2000). The chemical was also positive for MN induction in mice after ip treatment, and weakly positive for MN in rats, but negative for UDS (George & Westmoreland, 1991). It was negative in both acute and repeat dose liver MN tests (Takasawa et al, 2012). It was negative for transgenic mutations in the liver of mice treated by diet and topically. Low level DNA adduct formation was reported in Fischer-344 rat liver (Taningher et al, 1995). 2,6-Diaminotoluene dihydrochloride was not carcinogenic for male and female rats or for male and female mice after 2-year feeding (HSDB, 2003). The carcinogen 2,4-diaminotoluene produced a dose dependent increase in cell proliferation of approximately 10 – 20% in livers of

animals, whereas the noncarcinogen 2,6-diaminotoluene produced no increase in cell turnover compared to vehicle control. These results indicate a positive correlation between increased cell proliferation and hepatocarcinogenesis induced by these two isomers of diaminotoluene (HSDB, 2003). Based on the above analysis, 2,6-diaminotoluene would be expected not to induce comets in the stomach, and probably also not induce comets in liver.

81. In this validation trial, 8-hydroxyquinoline was negative for comets in liver but gave a reduction in % tail DNA in stomach. 8-Hydroquinoline was positive in Ames and in vitro CA tests. However, in vivo genotoxicity tests including UDS, MN and Comet assays all gave negative results in both rats and mice. 8-Hydroxyquinoline, given at 1500-3000 ppm in the feed of male and female rats and mice for 103 weeks, showed no evidence of carcinogenicity. On the other hand, the structurally related chemical quinoline is a specific and potent carcinogen in rat and mouse liver. 8-Hydroxyquinoline was metabolized to glucuronide and sulfate conjugates after iv administration (HSDB, 2002). Based on the above analysis, 8-hydroxyquinoline would not be expected to induce comets in stomach or liver, and the decrease in % tail DNA in the stomach of treated rats in this trial is unexpected and unexplained.

82. The remaining genotoxic non-carcinogens all gave negative results in both liver and stomach. Taking into account all known genotoxicity data, mode of action, and any route-, species- or tissue specificity, the expected outcomes for the remaining 4 chemicals, in terms of comet induction in liver or stomach of rats after oral dosing, can be summarised as follows:

83. 9-Aminoacridine hydrochloride monohydrate was positive in Ames and *in vitro* CA tests. No *in vivo* genotoxicity data was available. It is believed to be bacterial frame shift mutagen; it did not induce 6-thioguanine-resistant mutants in Chinese hamster V79 cells. There is no evidence that 9-aminoacridine is carcinogenic. It is a simple intercalating agent which will not bind covalently to DNA (O"Donovan, 1984). Based on the above, 9-aminoacridine would be expected to be negative for induction of comets in both stomach and liver.

84. *p*-Anisidine was positive in both the absence and presence of S9 in Ames, in vitro CA and mouse lymphoma assays. No in vivo genotoxicity data was available. *p*-Anisidine hydrochloride was not carcinogenic in male and female mice and female rats treated by the feed. Equivocal evidence was obtained in male rats (CPDB, HSDB, 2005). The compound was also negative in a short-term *in vivo* p53 heterozygous transgenic mouse carcinogenicity assay (Pritchard *et al*., 2003). Based on the above analysis, *p*-anisidine would not be expected to induce comets in stomach or liver of orally-dosed rats.

85. 5-Fluorouracil (5-FU) was not mutagenic in the Ames test but was positive for induction of CA in vitro. It induced MN in rats after ip or oral dosing, but was negative in a Comet assay in

mice treated by ip injection. Oral gavage administration to male rats induced positive Comet results after 3 hours exposure (C Beevers personal communication). 5-FU was also positive in a fetal liver MN test (Nakamura et al 1993). DNA damage, UDS and inhibition of DNA synthesis was observed in humans and test animals. Although 5-FU induced lung and hematopoietic system tumors in mice after 50 weeks of ip dosing, conventional 18-month mouse and 2-year rat studies using oral administration did not produce increased tumor incidence. Also, no evidence of carcinogenicity was observed in several animal studies following oral or iv administration of the drug for up to 1 year (IARC, 1987; HSDB, 2007) . 5-Fluorouracil is an antimetabolite of the pyrimidine analogue type. It is considered to be cell cycle-specific for the S phase of cell division. Activity results from its conversion to an active metabolite in the tissues, and includes inhibition of DNA and RNA synthesis (HSDB, 2007). It is postulated to inhibit thymidylate synthase activity, leading to a deficiency of thymidine and a decrease in DNA synthesis. Based on the above analysis, 5-FU might be expected to induce comets in the liver, but not in the stomach.

86. *p*-Phenylenediamine was positive in Ames and in vitro CA tests. However, it gave negative results in MN and UDS assays in rodents. There is no convincing evidence that dietary administration of p-phenylenediamine dihydrochloride was carcinogenic in rats or mice (HSDB, 2002). Based on the above analysis it is expected that *p*-phenylenediamine would not induce comets in either liver or stomach of rats by oral dosing.

7-3-3.Non-genotoxic carcinogens

87. One of seven non-genotoxic carcinogens tested, chloroform, induced a statistically significant (Dunnett‟s test) and dose dependant increase in % tail DNA in the liver in the 250 and 500 mg/kg/day groups. Histopathology for the liver showed the following changes: centrilobular hepatocellular single cell necrosis (grade: +), centrilobular hepatocellular necrosis (one of 5 rats, grade: +), centrilobular hepatocellular vacuolation (grade: + or ++), centrilobular hepatocellular enlargement/granular change (grade: + or ++), centrilobular inflammatory cell infiltration (grade: + or ++), and centrilobular hemorrhage (one of 5 rats, grade: ++) at 250 mg/kg/day; and centrilobular hepatocellular single cell necrosis (grade: +), centrilobular hepatocellular necrosis (one of 5 rats, grade: ++), centrilobular hepatocellular vacuolation (grade: + or ++), centrilobular hepatocellular enlargement/granular change (grade: + or ++), centrilobular inflammatory cell infiltration (grade: +), and centrilobular hemorrhage (one of 5 rats, grade: ++) at 500 mg/kg/day. Since serious cytotoxic findings such as moderate grade (++) hepatocellular necrosis and vacuolation followed by inflammatory cell infiltration and hemorrhage were observed with dose-dependency, the increased % tail DNA in the liver was considered to be related to cytotoxicity, and thus the final judgment was “Negative”, which was in agreement with the laboratory‟s own judgment. The remaining six non-genotoxic carcinogens did not induce any increases in % tail DNA in stomach or liver as judged by the individual

laboratory or statistical analysis. As a result, all test chemicals in this category were evaluated as "Negative" as the final judgment. No clear changes of % hedgehog were noted in any test chemicals in this category.

7-3-4.Non-genotoxic non-carcinogens

88. One of eight non-genotoxic non-carcinogens tested, *t*-butylhydroquinone (*t*-BHQ) induced a significant % tail DNA response in the liver, and the statistical judgment was "Increase" in the liver. However, the laboratory's own judgment was "Negative", because the increased % DNA in tail was within their historical control range which was established in testing conditions based on the validation study protocol (Brian Burlinson, personal communication). The final judgment for *t*-BHQ was "Positive" in the liver based on the statistical analysis results, but the VMT decided to note in this validation study report that the chemical was judged to be "Negative" in the testing facility. No changes of % hedgehog were noted with any of the test chemicals in this category. The remaining seven non-genotoxic non-carcinogens did not induce any increases in % tail DNA in stomach or liver as judged by the individual laboratory or statistical analysis. However, two chemicals (ethionamide and sodium chloride) were shown to induce some decreases in % tail DNA in the stomach according to statistical analysis. However, these were not considered to be biologically relevant (see discussion below), and the final judgment for both of these was negative. As a result, all test chemicals in this category were evaluated as "Negative" as the final judgment except for *t*-BHQ.

8. Discussion

89. The purpose of the 2nd step of the 4th phase (Phase 4-2) of this international validation study was to investigate the predictive capability of the *in vivo* rat alkaline comet assay against carcinogenicity of test chemicals. In general, the predictive capability of a screening genotoxicity assay for carcinogens is simply described as "positive sensitivity" for carcinogens, and "negative specificity" for non-carcinogens. Such a usual approach was applicable to this validation study data, but the VMT considered that the most appropriate approach to evaluate the predictive capability using these validation study results would be to take account of both genotoxic and carcinogenic properties of test chemicals, and any route-, species- or tissue-specificity i.e., focus on the concordance with expected Comet assay results in stomach and liver following oral dosing of rats determined before the validation study (see the section 6-4.). Therefore, we discuss the predictive capability of the *in vivo* rat alkaline Comet assay not only from the aspect of the four categories of test chemicals, i.e. genotoxic carcinogens, genotoxic non-carcinogens, non-genotoxic carcinogens, and non-genotoxic non-carcinogens, but also in terms of the expected outcomes for inducing comets on stomach and liver of rats following oral dosing..

90. For the genotoxic carcinogens, all were initially expected to be positive. However, 12 of the 19 genotoxic carcinogens tested were judged "Positive", six of 19 were "Negative", and one was "Equivocal". Since 13 of 19 genotoxic carcinogens were considered to show at least a positive alert, the positive sensitivity for genotoxic carcinogens would be simply calculated to be 13/19 (68%).

91. However, if route and tissue-specificity and mode of action are taken into account, as discussed in section 7-3-1, then a comparison of expected and actual Comet assay outcomes can be made:

Test chemical name	Comet outcome – liver		Comet outcome – stomach	
	Expected	Actual	Expected	Actual
2-Acetylaminofluorene	Positive	Negative	Negative	Negative
Acrylonitrile	Negative by oral dosing	Positive (final call) Negative (lab call)	Negative	Negative
o-Anisidine	Negative	Negative	Negative	Negative
Azidothymidine	Positive	Positive	Positive	Equivocal
Benzene	Negative	Negative	Negative	Negative
Busulfan	Decreased DNA migration	Negative	Decreased DNA migration	Negative
Cadmium chloride	Possible positive	Positive	Possible positive	Equivocal
p-Chloroaniline	Positive	Positive	Positive	Positive
Cisplatin	Probable negative	Positive	Probable negative	Negative
2,4-Diaminotoluene	Positive	Positive	Negative	Negative
1,2-Dibromomethane	Positive	Positive	Positive	Positive
1,3-Dichloropropene	Positive	Positive	Positive	Negative
1,2-Dimethylhydrazine dihydrochloride	Positive	Positive	Negative	Negative
Hydroquinone	Negative	Negative	Negative	Negative
Methyl methanesulfonate	Positive	Positive	Positive	Positive
N-Nitrosodimethylamine	Positive	Positive	Negative	Negative
4,4''-Oxydianiline	Probable negative	Negative	Probable negative	Decreased DNA migration
Sodium arsenite	Negative	Equivocal	Negative	Negative
Thioacetamide	Negative	Negative (due to cytotoxicity)	Positive	Positive

92. Thus, for the genotoxic carcinogens, 15/19 (78.9%) expected results were obtained in the liver, and 14/19 (73.7%) expected results were obtained in the stomach. This confirms the sensitivity and applicability of the assay with coded carcinogenic chemicals.

93. For the genotoxic non-carcinogens it was initially expected that they would give negative results. Based on this, the negative specificity for genotoxic non-carcinogens is calculated to be 83%, since 5 of 6 chemicals were judged to be "Negative". However, as discussed in section 7-3-2, some of these chemicals might be expected to induce DNA damage in certain tissues. If all available data including likely mode of action are taken into account, then a comparison of expected and actual Comet assay outcomes can be made:

Test chemical name	Comet outcome – liver		Comet outcome – stomach	
	Expected	Actual	Expected	Actual
9-Aminoacridine hydrochloride monohydrate	Negative	Negative	Negative	Negative
p-Anisidine	Negative	Negative	Negative	Negative
2,6-Diaminotoluene	Probable negative	Positive	Negative	Negative
5-Fluorouracil	Positive	Negative	Negative	Negative
8-Hydroxyquinoline	Negative	Negative	Negative	Decrease in DNA migration
p-Phenylenediamine	Negative	Negative	Negative	Negative

94. Thus, for the genotoxic non-carcinogens, 4/6 (66.7%) expected results were obtained in the liver, and 5/6 (83.3%) expected results were obtained in the stomach.

95. For the non-genotoxic carcinogens and the non-genotoxic non-carcinogens, all were expected to give negative comet responses in both liver and stomach. Since only *t*-butylhydroquinone gave a positive response (in liver) and all other chemicals in these 2 groups were negative in both tissues, the negative specificity for these 2 groups was calculated to be 93.3%, since 14 out of 15 chemicals were judged to be "Negative". If the 2 tissues are considered separately, for the non-genotoxic carcinogens and non-genotoxic non-carcinogens 14/15 (93.3%) expected results were obtained in the liver and 15/15 (100%) expected results were obtained in the stomach.

96. Considering the expected and actual results for both tissues for all 4 groups of chemicals, 33/40 (82.5%) expected results were obtained in the liver and 34/40 (85.0%) expected results were obtained in the stomach.

97. The high ability of the assay to deliver the expected positive and negative Comet assay outcomes, based on detailed knowledge of the compounds, their modes of action, and route-, species- and tissue specificity can be taken as convincing evidence that the applicability domain for the assay (namely to detect genotoxic and carcinogenic chemicals that induce DNA strand breakage) is appropriately defined.

98. Since this step of the validation study is the final stage of a series of pre-validation and validation efforts on the *in vivo* rat alkaline Comet assay, the second purpose of the validation should be discussed i.e. the use of the Comet assay as an alternative follow-up to the more commonly used *in vivo* rodent Unscheduled DNA Synthesis (UDS) assay. The genotoxic carcinogens used in this study included five UDS-positive chemicals, 2-acetylaminofluorene (2-AAF), 2,4-diaminotoluene, 1,2-dimethylhydrazine dihydrochloride, methyl methanesulfonate and *N*-nitrosodimethylamine, and four UDS-negative chemicals, acrylonitrile, *o*-anisidine, 1,3-dichloropropene and 4,4‟-oxydianiline. Four of the five UDS positives were judged to be "Positive" for induction of comets in the liver of rats in this study, but 2-AAF was "Negative". Two of the four UDS negatives, acrylonitrile and 1,3-dichloropropene were judged to be "Positive" in this study, although acrylonitrile was judged to be negative by the testing laboratory based on their historical control data. Although the number of chemicals tested is limited, the VMT concluded that Comet assay could be at least equal to or maybe more sensitive in detecting genotoxic carcinogens than the UDS assay.

99. In discussing the usefulness of the Comet assay in detecting genotoxic or carcinogenic chemicals, it should be taken into account that only the liver and the stomach (glandular stomach) were analyzed in this validation study because the validation study protocol was designed to screen for genotoxic and/or carcinogenic activity without consideration of the target organ specificity. The liver is the primary organ for the metabolism of absorbed chemicals, and the stomach is a site of first contact of chemicals after oral administration and these organs were recommended for screening purposes in the previous discussion in ICAW (Tice, et al., 2000). Interestingly, in this validation study, the following chemicals were judged to be "Positive" in the liver and/or the stomach, although both organs were not targets in the rodent carcinogenicity studies: acrylonitrile, azidothymidine, cadmium chloride, *p*-chloroaniline, and thioacetamide (Table 2). In addition, the VMT had previously decided, before starting this step of validation study, that target organ specificity of carcinogenicity might be considered in the interpretation of negative results from genotoxic carcinogens, and this was taken into account in determining the expected Comet assay results (see the sections 6-4 and 7-3-1.). The assay can surely be applied to detect DNA damage in other tissues, and rational decisions regarding the most appropriate tissues to sample should be based on all available knowledge at the time of starting the study.

100. The within- and/or between-laboratory variability of assay results was examined to some extent in the 1st to 3rd phase pre-validation studies conducted in four or five lead-laboratories, and the variability found in the early phases was reduced by modifications to the study protocol for subsequent phases. By using version 14 of the study protocol, good between-laboratory reproducibility was noted, and this was robustly confirmed in Phase 4-1 of this validation study conducted in 13 laboratories. The within/between-laboratory reproducibility was also evaluated in this study using negative and positive control group data. Good within-laboratory reproducibility was observed, indicating that the experiments were well controlled in each testing facility. Although slight between-laboratory variability was noted, the variation was within the range of data-acceptance criteria except for one negative control value of the liver in one laboratory. Those results indicate that consistent Comet assay results could be obtained within and/or between laboratories as far as the experiments are conducted with the version 14.2 study protocol.

101. Regarding cytotoxicity evaluation, histopathology was used in this validation study based on the previous consensus of the IWGT meeting (Hartmann, et al., 2003). Histopathology criteria for interpretation of positive findings in the Comet assay were originally proposed by the VMT based on histopathological findings with the negative test chemicals in the Comet assay and independent interpretation by each test facility. The interpretation method was then discussed and applied to each of the blinded test chemicals by all participants at the Kyoto meeting. As a result, increased % tail DNA in the liver observed with both thioacetamide and chloroform was interpreted to be related to severe cytotoxicity. Both chemicals are well-known hepatotoxicants, and the carcinogenic MoA of chloroform in the liver is reported to be due to cytotoxicity followed by regenerative hepatocyte proliferation (Maronpot, et al., 1989). Thus the interpretation of positive findings in the Comet assay as being an indirect consequence of liver toxicity based on histopathology findings was considered justified for chloroform. In contrast, it is recommended that positive findings in the Comet assay should be interpreted as relevant to *in vivo* genotoxicity even if weakly cytotoxic changes such as single cell death/necrosis are observed by histopathology, because many genotoxic carcinogens showing significant increases in % tail DNA in this validation study induced such slight cytotoxicity as observed by histopathological changes.

102. In contrast to histopathology, clear increases in % hedgehog cells were found with only three genotoxic carcinogens, 1,2-dimethylhydrazine dihydrochloride, methyl methanesulfonate, and *N*-nitrosodimethylamine. There were no relationships between positive judgments in the Comet assay or histopathological findings and increased % hedgehog cells, indicating that % hedgehog cells would not be a good indicator for cytotoxicity evaluation. Presumably,

increased % hedgehog cells might be an indicator of severe genotoxicity, because increased % hedgehog cells were only observed following treatment with the three above-mentioned genotoxic carcinogens that showed high increases in % tail DNA in this validation study, and also following treatment with *N*-methyl-*N*-nitrosourea, which also showed very high increases in % tail DNA in Phase 4-1 of the validation study.

103. The significance of decreased % tail DNA in the stomach, which was observed following treatment with acrylonitrile (genotoxic carcinogen), 4,4‟-oxydianiline (genotoxic carcinogen), 8-hydroxyquinoline (genotoxic non-carcinogen), ethionamide (non-genotoxic non-carcinogen), and sodium chloride (non-genotoxic non-carcinogen) is also worthy of discussion. Since those chemicals are not known to be cross-linking agents, and the typical cross-linking agents, busulfan and cisplatin, failed to decrease % tail DNA in this validation study, decreased % tail DNA found in this validation study would not appear to be indicative of a cross-linking genotoxic MoA. A possible explanation to interpret the significance of decreased % tail DNA observed with the 5 chemicals listed above may be that cytotoxic effects caused the decreased % tail DNA in the stomach. For example, acrylonitrile induced cytotoxicity in the stomach according to histopathological findings, and sodium chloride was selected for this validation study because it is a well-known cytotoxic agent for the stomach. Regarding a possible mechanism for the decreased % tail DNA, it was pointed out that, when cytotoxicity was observed in the stomach, the epithelial cells would be eliminated and then cells mainly recovered from the stomach for Comet analysis would be the basal cells which show lower values of % DNA in tail (Sachiko Kitamoto, personal communication at the Kyoto meeting). Since histopathology was not always examined for the stomach in this validation study - it was only done in the case of increased % tail DNA - further investigation of histopathology for the stomach would be needed to clarify the relationship between decreased % tail DNA in the stomach and the histopathological changes.

104. Based on the discussion above, the VMT has suggested that there may be two different aspects regarding the relationship between changes of % tail DNA and cytotoxicity, i.e., severe cytotoxicity would be expected to increase % tail DNA in the liver but might decrease it in the stomach. It is clear that any changes in % tail DNA would require very careful interpretation if they were seen alongside severe cytotoxic changes observed through histopathology. In such cases further investigation using lower doses or other genotoxicity tests, such as DNA-adduct formation or gene mutation assays with transgenic animals, may be required.

105. The use of historical control data is also worthy of discussion, especially for considering the biological significance when increased % DNA in tail is noted following treatment with a test chemical. This topic has already discussed in IWGT meetings (Butterworth, et al., 1995), and it has been suggested that the distribution of historical control data should be established with negative control data from at least 10 (preferably 20) independent experiments conducted

with the same study protocol and testing conditions (after technical maturation). Although the distribution of historical control data was not considered in the evaluation of results in this validation study, it would be practically possible to use the distribution in each testing facility, as long as it has been established based on the IWGT consensus, for the interpretation of the biological significance of increased % DNA in tail.

106. Only the liver and the stomach were selected for evaluation in this validation study but the possibility of application of the methodology to other organs needs to be discussed. The cell preparation methods used for the liver and the stomach would be basically applicable to other organs that can be classified as parenchymatous (i.e. similar to the liver) or hollow organs (i.e. similar to the stomach). Once cell suspensions have been prepared, the other technical procedures used in the Comet assay and described in the study protocol are, of course, applicable to all organs. The methods to prepare single cell suspensions were discussed in the IWGT and ICAW meetings (ICH, 2012, Tice, et al, 2000), and it has been already agreed that single cell suspensions can be obtained from any solid tissues by using various methods, e.g., mincing briefly with a pair of fine scissors (McNamee, et al., 2000) as performed in this validation study, incubation with digestive enzymes such as collagenase or trypsin (Tice, et al., 1991), or by pushing the tissue sample through a mesh membrane, as long as it can be demonstrated that the process leads to good cell survival and is not associated with inappropriate background levels of DNA damage (Tice, et al, 2000). The international consensus clearly supports applicability of the Comet assay protocol as developed in this validation study to any tissue, and not only to the liver and the stomach. When applied to other organs, it would be necessary to collect sufficient historical control data for each organ in each testing facility with vehicle controls and a positive control such as EMS (see the above paragraph about how to establish historical control data).

107. Finally, it is important to discuss how the *in vivo* rodent alkaline Comet assay should be conducted in order to obtain a toxicologically significant result. Many researchers have expressed concerns that the Comet assay may be susceptible to a high frequency of false-positive results. It has been noticeable throughout the progress of this validation effort that low % tail DNA values in the negative control group would sometimes lead to questionable (probably toxicologically insignificant) results when the increases in % tail DNA in treated groups were only marginally higher and still within the historical control distribution. Researchers using the Comet assay may believe that lower values in the negative control group (zero if possible) would be more effective in allowing the detection of very slight increases in % tail DNA in treated groups. However, such an approach would most likely lead to even more false-positive (toxicologically insignificant) results. In this validation study, there were no clearly false-positive results - even the non-genotoxic non-carcinogen *t*-butylhydroquinone, which gave a statistically significant comet response in the liver, was considered negative by the

testing lab oratory based on historical control data - and this would strongly indicate the usefulness of aiming to achieve measurable % tail DNA values in control groups (i.e. not aiming for zero) as has been described in the standardized study protocol and the data-acceptance criteria. The VMT would like to stress that the data-acceptance criteria used in this validation study should be strictly followed to obtain a toxicologically significant result with *in vivo* Comet assay. In order to satisfy the criteria, establishment of technical competence would be required as in all genotoxicity assays.

9. References

Ayers, K.M., Clive, D., Tucker, W.E., Hajian, G. & de Miranda, P. (1996a) Nonclinical toxicology studies with zidovudine: Genetic toxicity tests and carcinogenicity bioassays in mice and rats. Fundam. appl. Toxicol., 32, 148–158

Biswas R, Poddar S, Mukherjee A. Investigation on the genotoxic effects of long-term administration of sodium arsenite in bone marrow and testicular cells in vivo using the comet assay. J Environ Pathol Toxicol Oncol. 2007;26(1):29-37.

Brendler-Schwaab SY, Schmezer P, Liegibel U, Weber S, Michalek K, Tompa A, Pool-Zobel BL. Cells of different tissues for in vitro and in vivo studies in toxicology: compilation of isolation methods. Toxicol. In Vitro 1994; 8:1285-1302.

Burlinson B, Tice RR, Speit G, Agurell E, Brendler-Schwaab SY, Collins AR, Escobar P, Honma M, Kumaravel TS, Nakajima M, Sasaki YF, Thybaud V, Uno Y, Vasquez M, Hartmann A. Fourth International Workgroup on Genotoxicity Testing: result of the in vivo comet assay workgroup. Mutat. Res. 2007; 627:31-5.

Butterworth BE, Templin MV, Borghoff SJ, Conolly RB, Kedderis GL, Wolf DC. The role of regenerative cell proliferation in chloroform-induced cancer. Toxicol Lett. 1995; 82/83:23-26.

Celik A, Cömelekoğlu U, Yalin S. A study on the investigation of cadmium chloride genotoxicity in rat bone marrow using micronucleus test and chromosome aberration analysis. Toxicol Ind Health. 2005;21(10):243-8.

Chemical Carcinogenesis Research Information System, 1,2-Dimethylhydrazine dihydrochloride, CCRIS Record Number: 260, Last Revision Date: 19980702, http://toxnet.nlm.nih.gov/

George, E., Westmoreland, C., Evaluation of the in vivo genotoxicity of the structural analogues 2,6-diaminotoluene and 2,4-diaminotoluene using the rat micronucleus test and rat liver UDS assay, Carcinogenesis, 12 (1991) 2233-2237.

Gold LS. The Carcinogenic Potency Database (CPDP). Available at http://potency.berkeley.edu/cpdb.html (accessed 21 March, 2012).

Hartmann A, Agurell E, Beevers C, Brendler-Schwaab S, Burlinson B, Clay P, Collins A, Smith A, Speit G, Thybaud V, Tice RR. Recommendation for conducting the *in vivo* alkaline Comet assay. Mutagenesis 2003; 18:45-51.

IARC, Some antiviral and antineoplastic drugs, and other pharmaceutical agents, IARC monographs on the evaluation of carcinogenic risks to humans, volume 76, 2000. <http://monographs.iarc.fr/>

IARC monograph 100A, pp 37-44(2012)

ICH Guidance on genotoxicity testing and data interpretation for pharmaceuticals intended for human use S2(R1), ICH harmonized tripartite guideline, 2012.

Ishidate, M. Jr (ed), Data book of chromosomal aberration tests in vitro, LIC Inc., Tokyo, pp. 141, 1987.

Kadirvel R, Sundaram K, Mani S, Samuel S, Elango N, Panneerselvam C. Supplementation of ascorbic acid and alpha-tocopherol prevents arsenic-induced protein oxidation and DNA damage induced by arsenic in rats. Hum Exp Toxicol. 2007 ;26(12):939-46.

Kirkland D, Aardema M, Henderson L, Müller L. Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens. I. Sensitivity, specificity and relative predictivity. Mutat. Res. 2005; 584:1-256.

Kirkland D, Speit G. Evaluation of the ability of a battery of three *in vitro* genotoxicity tests to discriminate rodent carcinogens and non-carcinogens III. Appropriate follow-up testing *in vivo*. Mutat. Res. 2008a; 654:114-132.

Kirkland D, Kasper P, Müller L, Corvi R, Speit G. Recommended list of genotoxic and non-genotoxic chemicals for assessment of the performance of new or improved genotoxicity tests: A follow-up to an ECVAM workshop. Mutat. Res. 2008b; 653:99-108.

Madle S, Dean SW, Andrae U, Brambilla G, Burlinson B, Doolittle DJ, Furihata C, Hertner T, McQueen CA, Mori H. Recommendations for the performance of UDS tests in vitro and in vivo. Mutat Res. 1994; 312(3):263-85.

Maronpot RR, Pitot HC, Peraino C. Use of rat liver altered focus models for testing chemicals that have completed two-year carcinogenicity studies. Toxicol Pathol. 1989; 17(4 Pt 1):651-62.

Mavournin, K.H., Blakey, D.H., Cimino, M.C., Salamone, M.F., Heddle, J.A., The in vivo micronucleus assay in mammalian bone marrow and peripheral blood. A report of the U.S. Environmental Protection Agency Gene-Tox Program, Mutat Res, 239 (1990) 29-80.

McGregor D. Hydroquinone: an evaluation of the human risks from its carcinogenicity and mutagenic properties. Crit Rev Toxicol. 2007; 37(10):887-914.

McKenna DJ, Gallus M, McKeown SR, Downes CS, McKelvey-Martin VJ. Modification of the alkaline Comet assay to allow simultaneous evaluation of mitomycin C-induced DNA cross-link damage and repair of specific DNA sequences in RT4 cells. DNA Repair (Amst). 2003; 2(8):879-90.

McNamee JP, McLean JRN, Ferrarotto CL, Bellier PV. Comet assay: rapid processing of multiple samples. Mutat. Res. 2000; 466:63-69.

Meng Q, Su T, Olivero OA, Poirier MC, Shi X, Ding X, Walker VE. Relationships between DNA incorporation, mutant frequency, and loss of heterozygosity at the TK locus in human lymphoblastoid cells exposed to 3'-azido-3'-deoxythymidine. Toxicol Sci. 2000 ;54(2):322-9.

Nakajima M, Ueda M, Yamakage K, Nakagawa Y, Nakagawa M, Ohyama W, Omori T, Asano N, Hayashi M, Uno Y. Tissue sample preparation for *in vivo* rodent alkaline comet assay. Genes Environ. 2012; 34:50-4.

Nakamura M, Fort FL, Kikuchi Y. Fetal liver micronucleus assay in mice of 5-fluorouracil and related compounds. Mutat Res. 1993 ;291(1):29-34.

Natl. Toxicol. Program Tech Rep. Ser. 1978; 89.

Natl Toxicol Program Tech Rep Ser. 1980; 205:1-131.

Natl. Toxicol. Program Tech Rep. Ser. 1986; 289:1-277.

Natl. Toxicol. Program Tech Rep Ser. 1989; 366:1-248.

NTP Database Search Home Page, CAS 30516-87-1, Study ID 860655, http://tools.niehs.nih.gov/ntp_tox/index.cfm

NTP Database Search Home Page, CAS 30516-87-1, Study ID 912603, http://tools.niehs.nih.gov/ntp_tox/index.cfm

Nesslany F, Zennouche N, Simar-Meintières S, Talahari I, NKili-Mboui EN, Marzin D. *In vivo* Comet assay on isolated kidney cells to distinguish genotoxic carcinogens from epigenetic carcinogens or cytotoxic compounds. Mutat. Res. 2007; 630:28-41.

O'Donovan, M.R. , Mutagenicity of some intercalating agents in Chienese hamster V79 cells, Mutat. Res. 137 (1984) 117-122.

Pritchard JB, French JE, Davis BJ, Haseman JK. The role of transgenic mouse models in carcinogen identification. Environ Health Perspect. 2003;111(4):444-54.

Rogers AM, Back KC. Comparative mutagenicity of hydrazine and 3 methylated derivatives in L5178Y mouse lymphoma cells. Mutat Res. 1981 ;89(4):321-8.

Rothfuss, A., O'Donovan, M., De Boeck, M., Brault, D., Czich, A., Custer, L., Hamada, S., Plappert-Helbig, U., Hayashi, M., Howe, J., Kraynak, A.R., van der Leede, B.J., Nakajima, M., Priestley, C., Thybaud, V., Saigo, K., Sawant, S., Shi, J., Storer, R., Struwe, M., Vock, E., Galloway, S., Collaborative study on fifteen compounds in the rat-liver Comet assay integrated into 2- and 4-week repeat-dose studies, Mutat Res, 702 (2010) 40-69.

Rothfuss A, Honma M, Czich A, Aardema MJ, Burlinson B, Galloway S, Hamada S, Kirkland D, Heflich RH, Howe J, Nakajima M, O'Donovan M, Plappert-Helbig U, Priestley C, Recio L, Schuler M, Uno Y, Martus HJ. Improvement of in vivo genotoxicity assessment: combination of acute tests and integration into standard toxicity testing. Mutat Res. 2011; 723(2):108-20.

Sasaki, Y.F., Fujikawa, K., Ishida, K., Kawamura, N., Nishikawa, Y., Ohta, S., Satoh, M., Madarame, H., Ueno, S,. Susa, N., Matsusaka, N., Tsuda, S., The alkaline single cell gel electrophoresis assay with mouse multiple organs: results with 30 aromatic amines evaluated by the IARC and U.S. NTP, Mutat Res, 440 (1999) 1-18

Sasaki YF, Sekihashi K, Izumiyama F, Nishidate E, Saga A, Ishida K, Tsuda S. The comet assay with multiple mouse organs: comparison of comet assay results and carcinogenicity with 208 chemicals selected from the IARC monographs and U.S. NTP Carcinogenicity Database. Crit. Rev. Toxicol. 2000; 30:629-799.

Seifried HE, Seifried RM, Clarke JJ, Junghans TB, San RH. A compilation of two decades of mutagenicity test results with the Ames Salmonella typhimurium and L5178Y mouse lymphoma cell mutation assays. Chem Res Toxicol. 2006 May;19(5):627-44.

Sekihashi K, Yamamoto A, Matsumura Y, Ueno S, Watanabe-Akanuma M, Kassie F, Knasmüller S, Tsuda S, Sasaki YF. Comparative investigations of multiple organs of mice and rats in the comet assay. Mutat. Res. 2002; 517:53-74.

Séverin I, Jondeau A, Dahbi L, Chagnon MC. 2,4-Diaminotoluene (2,4-DAT)-induced DNA damage, DNA repair and micronucleus formation in the human hepatoma cell line HepG2. Toxicology. 2005 Sep 15;213(1-2):138-46.

Stiborová M, Miksanová M, Sulc M, Rýdlová H, Schmeiser HH, Frei E. Identification of a genotoxic mechanism for the carcinogenicity of the environmental pollutant and suspected human carcinogen *o*-anisidine. Int. J. Cancer. 2005; 116(5):667-78.

Takasawa H, Takashima R, Hattori A, Narumi K, Kawasako K, Morita T, Hayashi M, Hamada S. Development of a repeated-dose liver micronucleus assay using adult rats (II): Further investigation of 1,2-dimethylhydrazine and 2,6-diaminotoluene. In press, Mutat Res. 2012

Taningher M, Peluso M, Parodi S, Ledda-Columbano GM, Columbano A. Genotoxic and non-genotoxic activities of 2,4- and 2,6-diaminotoluene, as evaluated in Fischer-344 rat liver. Toxicology. 1995 May 5;99(1-2):1-10.

Tice RR, Andrews PW, Hirai O, Singh NP. The single cell gel (SCG) assay: an electrophoretic technique for the detection of DNA damage in individual cells. In Witmer CR, Synder RR, Jollow DJ, Kalf GF, Kocsis JJ, Sipes IG (eds), Biological Reactive Intermediates IV, Molecular and Cellular Effects and their Impact on Human Health. Plenum Press, New York, NY, 1991, pp.157-164.

Tice RR, Yager JW, Andrews P, Crecelius E. Effect of hepatic methyl donor status on urinary excretion and DNA damage in B6C3F1 mice treated with sodium arsenite. Mutat Res. 1997 ;386(3):315-34.

Tice RR, Agurell E, Anderson D, Burlinson B, Hartmann A, Kobayashi H, Miyamae Y, Rojas E, Ryu JC, Sasaki YF. Single cell gel/Comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ. Mol. Mutagen. 2000; 35:206-21.

Torres SM, Walker DM, Carter MM, Cook DL Jr, McCash CL, Cordova EM, Olivero OA, Poirier MC, Walker VE. Mutagenicity of zidovudine, lamivudine, and abacavir following in vitro exposure of human lymphoblastoid cells or in utero exposure of CD-1 mice to single agents or drug combinations. Environ Mol Mutagen. 2007;48(3-4):224-38

US.EPA: Reproductive and Cancer Hazard Assessment Section, Office of Environmental Health Hazard Assessment, California Environmental Protection Agency. Evidence on the carcinogenicity of 2-aminofluorene, draft. 1998.

US. EPA-IRIS. Available at http://www.epa.gov/iris/subst/0276.htm (accessed 21 March, 2012).

Vikram, A., Tripathi, D.N., Pawar, A.A., Ramarao, P., Jena, G.B., Pre-bled-young-rats in genotoxicity testing: A model for peripheral blood micronucleus assay, Regulatory Toxicology and Pharmacology 52 (2008) 147–157.

Phase 4.2 Appendix 1

INTERNATIONAL VALIDATION OF THE *IN VIVO* RODENT ALKALINE COMET ASSAY FOR THE DETECTION OF GENOTOXIC CARCINOGENS (VERSION 14.2)

Issued by: the Validation Management Team (VMT)
Date: November 30, 2009 revised

A. PURPOSE OF THIS DOCUMENT

This document is provided to clarify the conduct of an international validation study to evaluate the ability of the *in vivo* rodent alkaline Comet assay to identify genotoxic carcinogens, as a potential replacement for the *in vivo* rodent hepatocyte unscheduled DNA synthesis (UDS) assay. This document represents the final study protocol developed as a result of the collaboration efforts of the participating testing facilities and the VMT. Each testing facility will develop a study protocol based on the information provided in this document.

B. ASSURANCE OF DATA QUALITY

The study will be conducted in facilities that are Good Laboratory Practice compliant. Consistency between raw data and a final report is the responsibility of each testing facility. The VMT may review the data for accuracy, if deemed necessary.

C. ANIMAL WELFARE AND 3Rs

Appropriate national and/or international regulations on animal welfare should be followed. The 3Rs-principle for experimental animal use should be considered for determining the experimental design.

D. TESTING PROCEDURE

1. MATERIALS AND METHODS

1.1 Test substances and positive/negative controls

1.1.1 Test substance

With the exception of ethyl methanesulfonate (EMS), test substances will be supplied to each testing facility by the VMT. When coded substances are supplied, appropriate safety information will be provided in a sealed envelope to be opened only by an appropriate individual within the organization who is not involved in the study and/or in the case of an emergency. If opened, appropriate documentation and justification will need to be provided to the VMT.

1.1.2 Test substance preparation

Each test substance will be dissolved or suspended with an appropriate solvent/vehicle just before administration (see section 1.1.4.).

1.1.3 Positive control

EMS (CAS No. 62-50-0); the source and lot number to be used will be provided by the VMT. EMS will be dissolved in physiological saline just before administration (within 2 hours).

1.1.4 Negative control (solvent/vehicle)

Solvents/vehicles for test substance preparation will be used as negative controls. An appropriate solvent/vehicle for a test substance may be indicated by the VMT. In the absence of instruction from the VMT, an appropriate solvent/vehicle will be chosen for each test substance by the testing facility in the following order: physiological saline, 0.5% w/v sodium carboxymethylcellulose aqua solution, corn oil.

1.2 Test animals

1.2.1 Species

Although either rats or mice can be used in this assay, the validation study will use rats. The rat is the species most commonly used in toxicological studies and is the preferred species in the *in vivo* rodent hepatocyte UDS assay.

1.2.2 Sex

In order to allow for a direct comparison with the rat hepatocyte UDS assay, males will be used.

1.2.3 Strain

Rat: Crl:CD (SD)

1.2.4 Source

Charles River Laboratories, Inc.

1.2.5 Age

At the time of purchase: 6-8 weeks of age (body weight 150 g - 320 g)
At the time of dosing: 7-9 weeks of age

1.2.6 Body weight

The weight variation of animals should be +/- 20% of the mean weight at the time of dosing.

1.2.7 Number of animals in each dose group at each sampling time

Five males (see note 1).

1.2.8 Animal maintenance

Animals will be reared under appropriate housing and feeding conditions according to the standard operating procedures (SOP) in each testing facility, consistent with Section C “Animal Welfare".

1.2.8.1 Diet

Animals will be fed *ad libitum* with a commercially available pellet diet.

1.2.8.2 Water

Animals will be given free access to tap water *ad libitum*.

1.2.9 Animal quarantine and acclimation

Animals will be quarantined and acclimated for at least 5 days prior to the start of the study, according to SOPs in each testing facility. Only healthy animals approved by the Study Director and/or the Animal Facility Veterinarian will be used.

1.2.10 Animal identification and group assignment

Animals will be identified uniquely and assigned to groups by randomization on the basis of body weight according to the SOP in each testing facility.

1.3 Preparation of Comet assay solutions

The following solutions will be prepared, consistent with laboratory SOPs, unless otherwise specified (see note 2).

1.3.1 1.0-1.5% (w/v) standard agarose gel for the bottom layer (if used)

Regular melting agarose will be dissolved at 1.0-1.5% (w/v) in Dulbecco's phosphate buffer (Ca^{++}, Mg^{++} free and phenol free) by heating in a microwave.

1.3.2 0.5 % (w/v) low-melting agarose (Lonza, NuSieve GTG Agarose) gel for the cell-containing layer and, if used, a top layer

Low-melting agarose will be dissolved at 0.5% (w/v) in Dulbecco's phosphate buffer (Ca^{++}, Mg^{++} free and phenol free) by heating in a microwave. During the study this solution will be kept at 37-45°C and discarded afterward.

1.3.3 Lysing solution

The lysing solution will consist of 100 mM EDTA (disodium), 2.5 M sodium chloride, and 10 mM tris hydroxymethyl aminomethane in purified water, with the pH adjusted to 10.0 with 1 M sodium hydroxide and/or hydrochloric acid. This solution may be refrigerated at <10°C until use. On the same day of use, 1 % (v/v) of triton-X100 and 10 % (v/v) DMSO will be added to this solution and the complete lysing solution will be refrigerated at <10°C for at least 30 minutes prior to use.

1.3.4 Alkaline solution for unwinding and electrophoresis

The alkaline solution consists of 300 mM sodium hydroxide and 1 mM EDTA (disodium) in purified water, pH >13. This solution will be refrigerated at <10°C until use. The pH of the solution will be measured just prior to use.

1.3.5 Neutralization solution

The neutralization solution consists of 0.4 M tris hydroxymethyl aminomethane in purified water, pH 7.5. This solution will be either refrigerated at <10°C or stored consistent with

manufacturer"s specifications until use.

1.3.6 Mincing buffer

The mincing buffer consists of 20 mM EDTA (disodium) and 10% DMSO in Hank"s Balanced Salt Solution (HBSS) (Ca^{++}, Mg^{++} free, and phenol red free if available), pH 7.5 (DMSO will be added immediately before use). This solution will be refrigerated at <10°C until use.

1.3.7 Staining solution

The fluorescent DNA stain is SYBR Gold (Invitrogen-Molecular Probes), prepared and used according to the manufacturer"s specifications.

1.4 Comet assay procedure

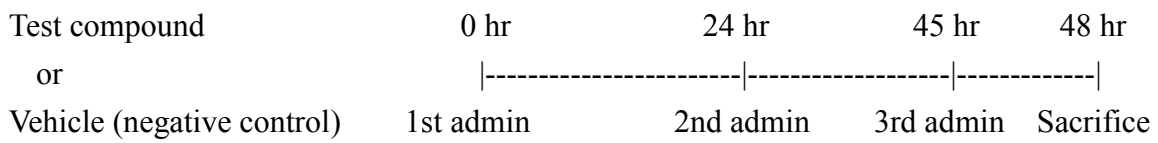
1.4.1 Experimental design

Compound	Dose (mg/kg/day)	Number of animals (see note 1)
Vehicle (negative control)	0	5
EMS (positive control)	200	5
Test compound	Low (1/4 of high)	5
Test compound	Medium (1/2 of high)	5
Test compound	High*	5

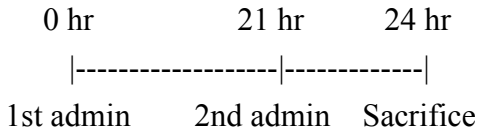
*High dose selection (see note 3): in general, in the absence of VMT directions, the high dose level of a test compound will be selected as the dose producing signs of toxicity such that a higher dose level, based on the same dosing regimen, would be expected to produce mortality, or an unacceptable level of animal distress. Selection of doses will be based on the toxicity of the test substance but will not exceed 2000 mg/kg/day.

1.4.2 Administration to animals

The test substance will be administered three times orally by gavage, 24 and 21 hours apart, i.e. the second administration is 24 hours after the first administration, and the third administration is 21 hours after the second administration (at 3 hours before animal sacrifice). EMS will be administered twice orally by gavage at 24 hours and 3 hours before animal sacrifice. The administration regimes are summarized in a figure below; this protocol enables us to integrate the comet and micronucleated erythrocyte assay into one assay (see note 4). The dosage volume will be 0.1 mL per 10 g body weight in rats on the basis of the animal weight just before administration.



EMS (positive control)



1.4.3 Measurement of body weight and examination of animal conditions

Individual body weights will be measured in accordance with local SOPs and just prior to administration (the weight at this time will be used to determine the volume of each substance administered) and at the time of termination. The clinical signs of the animals will be observed from just after dosing to just before tissue removal with an appropriate interval according to the SOP in each testing facility.

1.4.4 Tissue sampling

Animals will be humanely killed at 3 hours after third administration of a test substance and at 3 hours after second treatment of EMS, consistent with Section C "Animal Welfare and 3Rs". The stomach and the liver will be removed (see note 5). Tissues will be placed into ice-cold mincing buffer, rinsed sufficiently with the cold mincing buffer to remove residual blood (more rinses would likely be needed if exsanguination is not used), and stored on ice until processed. For histopathology, samples will be obtained from the same liver lobe, and from a minimal possible area of stomach.

1.4.5 Preparation of single cells

Single cell preparation should be done within one hour after animal sacrifice (see note 6). The liver and the stomach will be processed as follows:

Liver: A portion of the left lateral lobe of the liver will be removed and washed in the cold mincing buffer until as much blood as possible has been removed (see note 7). The portion will be minced with a pair of fine scissors to release the cells. The cell suspension will be stored on ice for 15-30 seconds to allow large clumps to settle (or, the cell suspension will be strained through a Cell Strainer to remove lumps and the remaining suspension will be placed on ice), and the supernatant will be used to prepare comet slides.

Stomach: The stomach will be cut open and washed free from food using cold mincing buffer. The forestomach will be removed and discarded. The glandular stomach will be then placed into cold mincing buffer and incubated on ice for from 15 to 30 minutes. After incubation, the surface epithelia will be gently scraped two times using the a scalpel blade or a Teflon scrapper. This layer will be discarded and the gastric mucosa rinsed with the cold mincing buffer. The stomach epithelia will be carefully scraped 4-5 times (or more, if necessary) with a scalpel blade or Teflon scrapper to release the cells. The cell suspension will be stored on ice for 15-30 seconds to allow large clumps to settle (or, the cell suspension will be strained with a Cell Strainer to remove clumps and the remaining suspension will be placed on ice), and samples of the supernatant used to prepare comet slides.

1.4.6 Slide preparation

Slide preparation should be done within one hour after single cell preparation (see note 6). Comet slides will be prepared using laboratory specific procedures. The volume of the cell suspension added to 0.50% low melting agarose to make the slides will not decrease the percentage of low melting agarose by more than 10% (i.e., not below 0.45%) .

1.4.7 Lysis

Once prepared, the slides will be immersed in chilled lysing solution overnight in a refrigerator under a light proof condition (see note 6). After this incubation period, the slides will be rinsed in purified water or neutralization solution to remove residual detergent and salts prior to the alkali unwinding step.

1.4.8. Unwinding and electrophoresis

Slides will be randomly placed onto a platform of submarine-type electrophoresis unit and the electrophoresis solution added. A balanced design will be used (see note 8). The electrophoresis solution will be poured until the surfaces of the slides are completely covered with the solution. The slides will be left to be unwind for 20 minutes. Next, the slides will be electrophoresed at 0.7 V/cm for at least 20 minutes, with a constant voltage at approximately 300 mA (see note 9). The current at the start and end of the electrophoresis period should be recorded. The temperature of the electrophoresis solution through unwinding and electrophoresis should be maintained at a constant temperature <10°C . The temperature of the electrophoresis solution at the start of unwinding, the start of electrophoresis, and the end of electrophoresis should be recorded.

1.4.9. Neutralization and dehydration of slides

After completion of electrophoresis, the slides will be immersed in the neutralization buffer for at least 5 minutes. All slides will be dehydrated by immersion into absolute ethanol (≥99.6%) for at least 5 minutes if slides will not be scored soon, allowed to air dry, and then stored until scored at room temperature, protected from humidity > 60 %. Once scored, slides should be retained and stored under low humidity conditions (e.g., in a desiccator) for potential rescoring.

1.4.10. DNA staining, comet visualization and analysis

Coded slides will be blind scored according to laboratory specific SOPs. The slides will be stained with SYBR Gold according to manufacturer‟s specifications. The comets will be measured via a digital (e.g. CCD) camera linked to an image analyzer system using a fluorescence microscope at magnification of 200X. For each sample (animal/tissue), fifty comets per slide will be analyzed, with 2 slides scored per sample (see note 10). Approximately 10 areas/slide should be observed at 5 cells or less/field (see note 11), taking care to avoid any selection bias, overlap counting of cells, and edge areas of slides. Heavily damaged cells exhibiting a microscopic image (commonly referred to as hedgehogs) consisting of small or non-existent head and large, diffuse tails will be excluded from data collection if the image analysis system cannot properly score them (see note 12). However, the frequency of such

comets should be determined per sample, based on the visual scoring of 100 cells per sample. The comet endpoints collected will be % tail DNA, tail length in microns measured from the estimated edge of the head region closest to the anode (see note 13), and, if possible for a particular image analysis system, Olive tail moment [= a measure of tail length (a distance between a center of head mass and a center of tail mass; microns) X a measure of DNA in tail (% tail DNA/100): Olive et al., 1990]. (see note 14)

1.4.11. Histopathology

When a positive Comet assay response is obtained for a tissue, a sample histopathological assessment will be conducted to evaluate for the presence of apoptotic and/or necrotic cells according to the SOP in each testing facility.

2. STATISTICS

Different approaches for data analysis have been proposed for comet data generated across a range of test substance dose levels (Lovell et al. 1999; Hartmann et al. 2003; Wiklund and Agurell 2003). The primary endpoint of interest for DNA migration is the % tail DNA. In addition, the distribution of migration patterns among cells within an animal will be considered. The percentage of "hedgehogs" will also be evaluated as a function of treatment. The unit of analysis for a specific tissue is the individual animal.

In data analysis process of this validation study, three conceptual key terms, i.e. "Endpoint", "Estimate", and "Effect" are defined and used. Briefly, "Endpoint" is defined as individual observed values for a parameter such as % DNA in tail. "Estimate" is defined as a mean calculated with values of a particular "Endpoint" in each animal. "Effect" is defined as difference of an average of "Estimate" between a negative control group and a treatment group (see note 15). Dunnett"s test (two-sided, $P<0.05$) and linear Trend test (two-sided, $P<0.05$) will be applied to "Effect" to judge positive or negative as assay results. For the positive control group, Student"s t-test (one-sided, $P<0.025$) will be applied to the "Effect".

3. DATA AND REPORTING

3.1 Treatment of results

Individual animal data and group summaries will be presented in a fixed tabular form that will be provided from the VMT.

3.2 Evaluation and interpretation of results

A positive response is defined as a statistically significant change in the % tail DNA in at least one dose group in comparison with the vehicle control value using Dunnett"s test (two-sided, $P<0.05$) as well as a statistically significant linear Trend test (two-sided, $P<0.05$). A negative response is defined as the statistically nonsignificant change in both Dunnett"s test and the linear Trend test, and an equivocal response is defined as the statistically significant change in

either of Dunnett"s test or the linear Trend test. The positive control should produce a statistically significant increase in Student"s t-test (one-sided, P<0.025), and if not, the study data will not be acceptable. Where a positive response is obtained in a test substance group, the investigator(s) will assess the possibility that a cytotoxic rather than a genotoxic effect is responsible based on the percentage of "hedgehogs" and histopathology (see note 16). Positive results indicate that the test substance induce DNA damage in the target tissue(s) investigated. Negative results indicate that, under the test conditions used, the test substance does not induce DNA damage *in vivo* in the tissue(s) evaluated.

3.3 Study report

The study report from each testing facility will at least include the following information:

3.3.1 Test substance and positive/negative controls

Identification; Chemical Abstracts Service Registry number (when available); supplier, lot number and purity (when available); physiochemical properties relevant to the conduct of the study, if known; justification for choice of vehicle; and solubility and stability of the substances in the solvent/vehicle, if known.

3.3.2 Test animals

Species/strain used; number, age and sex of animals; source, housing conditions, quarantine and acclimation procedure, and animal identification and group assignment procedure; individual weight of the animals on the day of receipt, at the end of the acclimation period, and before administration (at the time of grouping), including body weight range, mean and standard deviation for each group; and choice of tissue(s) and justification.

3.3.3 Reagents to prepare reagent solutions

Identification; supplier; lot number; and time limit for usage if known.

3.3.4 Test conditions

Data from range-finding study, if conducted; rationale for dose level selection; details of test substance preparation; details of the administration of the test substance; methods for verifying that the test substance reached the general circulation or target tissue, if applicable; details of food and water quality; detailed description of treatment and sampling schedules; method of measurement of toxicity, including histopathology; detailed methods of single cell preparation; method of slide preparation, including duration between tissue sampling and slide preparation, agarose concentration, lysis conditions (duration for lysis, etc.), alkali conditions and pH, alkali unwinding time and temperature, electrophoresis conditions (pH, V/cm, mA, and temperature at the start of unwinding and the start and the end of electrophoresis) and staining procedure; criteria for scoring comets and number of comets analyzed per slide, per tissue and per animal; evaluation criteria; criteria for considering studies as positive, negative or equivocal.

3.3.5 Results

Signs of toxicity, including histopathology in the appropriate tissue(s) if applicable; individual

and mean values for DNA migration (and ranges) and % hedgehogs in individual tissue, animal, and group; concurrent positive and negative control data; and statistical evaluation.

3.3.6 Discussion of the results and/or conclusion, as appropriate.

4. ARCHIVES AND REVIEW

The study report and all raw data (including slide samples and image data) from this study will be retained according to the SOP in each testing facility. All raw data will be submitted to the management team for review if required.

5. NOTES

5.1 We evaluated the data of the 3rd phase validation studies as to whether or not fewer (two, three or four) animals were sufficient in the positive control group to show a statistically significant increase in the Effect (difference) with a one-tailed student‟s t-test ($P<0.025$). The analysis results were presented and discussed at the Florence meeting held on August 25-26, 2009, and the participants felt that the reduction of animal number would be possible but the slight decrease in the statistic power might require additional experiments and result in the increase in animal usage. Thus the VMT decided to continue using five animals as the positive control in this validation effort. We may need to further investigate the appropriate number of animals/group afterwards based upon power calculation.

5.2 We will likely need to specify shelf life for some solutions as we reconcile lab-specific protocols.

5.3 The VMT extensively discussed at the Osaka meeting held on Feb. 4-6, 2009 how a preliminary dose-finding study should be done to choose an appropriate high dose level, because selection of a suitable high dose would be closely related to the sensitivity/specificity of genotoxicity assays in general. The VMT decided to request each facility to submit its own protocol for dose-selection, and the VMT will review them and then direct each facility to use its own protocol as it is or to follow a dose-finding study protocol recommended by the VMT.

5.4 When following the regimen for EMS as a positive control, micronucleus (MN) induction will be detected in bone marrow but not in peripheral blood. To also detect MN induction in peripheral blood, it would be necessary to administer EMS as well as the other test chemicals three times. It was also pointed out at the Florence meeting (August, 2009) that four times administration of test chemicals excluding the positive control, EMS, would be needed if we expect to detect micronuclei in the peripheral blood.

5.5 In this validation study, Comet analysis for the liver and the stomach will be conducted. Comet analysis along with MN for the bone marrow and/or the peripheral blood are optional in this validation study.

5.6 At the Florence meeting, it was pointed out that the duration of tissue sampling should be kept to a set time (e.g. within 10 min) and the duration for lysis should be controlled, in order to obtain more stable negative control values. The VMT considers that such action would be

preferred and recommended but not required of participant laboratories because the feasibility would depend on the performance of each laboratory. To further address this issue, the duration of tissue sampling and the duration for lysis should be recorded in the study report of each facility.

5.7 The size of the liver portions will be at the discretion of the laboratory, because there is no recommendation for standardizing this step.

5.8 In each electrophoresis run, there should be the same number of slides from each animal in the study; see Attachment 1, an example of how to keep track of each slide during each electrophoresis run. Each laboratory will need to provide its own electrophoresis box chart, as different boxes can accommodate different numbers of slides.

5.9 Under those electrophoresis conditions, it is expected that an average DNA migration obtained in the negative control group will be 1-8% tail DNA for the liver, and 1-20% tail DNA for the stomach. These ranges were set based on the analysis with negative control data from the 2nd and 3rd phase validation studies, i.e. the average +/- 3XS.D. values were as follows in the 2nd and 3rd phase validation studies, respectively: 3.8+/-4.8 (n=15 from 5 labs) and 3.1+/-3.9 (n=12 from 4 labs) in the liver, and 12.5+/-6.9 (n=12 from 4 labs) and 8.8+/-9 (n=10 from 4 labs) in the stomach. The reason why the lowest value is set at 1 is to be able to detect a significant decrease in % DNA in the tail. The decrease in DNA migration is expected for cross-linkers, and if such agents are intended to be detected using the Comet assay then a decrease in migration would be easier to detect when the negative control value is at the higher end of the acceptable range. If the negative control average deviates from the range, the duration of electrophoresis will be adjusted to achieve this range.

5.10 An investigation was conducted to compare with two slides/animal and three slides/animal about some data of the 3rd phase validation study, and the result was presented and discussed at the Florence meeting. As there was no difference between them as far as the present analysis method was used, the VMT decided to use two slides/animal.

5.11 In order to obtain suitable areas for observation, dilution of cell suspension may be required during the single cell preparation process.

5.12 This instruction indicates that if a comet is analyzable by the software program then it should be analyzed. However the following cases will be excluded from the analysis: a) analyzable but the recognition by software is considered incorrect (e.g. the automatic recognition of nucleus center is shifted); and b) the staining of nucleus and/or migration is considered poor. At the Florence meeting, more detailed analysis methods were discussed and agreed to, i.e. cells should be classified into three categories, scorable, non-scorable and hedgehog, and also scorable cells with a 90% or more DNA in the tail should not be adopted as part of the data for analysis. The VMT will prepare a color atlas to instruct how to distinguish comet and hedgehog.

5.13 „Tail length" is defined as „Tail migration" in some image analyzers such as Comet IV.

5.14 At the Atagawa meeting held on March 13-14, 2008, there was discussion about the need

to collect data on tail length and Olive tail moment in this validation study. Again, there was brief discussion about this point at the Osaka meeting. The consensus was that % DNA in tail seems to be a sufficient endpoint for validation and therefore these parameters would no longer be analyzed statistically. However, data on tail length and tail moment will continue to be collected in this validation study in case there is a reason to analyze these data in the future.

5.15 Effect (difference) seems to be more suitable for revealing variation between labs than Effect (ratio), which was pointed out at Osaka meeting in the discussion of the data of 3rd phase validation study.

5.16 At the present moment, there is no evident data on the consistency between the percentage of "hedgehogs" and histopathology. In this validation study, histopathology will be used as a primary endpoint to evaluate cytotoxicity, although both of the data will be collected for further analysis on the consistency between the percentage of "hedgehogs" and histopathology.

6. REFERENCES

Burlinson B, et al., Fourth International Workgroup on Genotoxicity Testing: result of the in vivo comet assay workgroup. Mutation Res., 627, 31-35, 2007.

Collins AR, et al., Direct enzymatic detection of endogenous oxidative base damage in human lymphocyte DNA. Carcinogenesis, 14, 1733-1735, 1993.

Hartmann A, et al., Recommendation for conducting the *in vivo* alkaline Comet assay. Mutagenesis, 18(1), 45-51, 2003.

Lovell DP, G Thomas G, R Dubow., Issues related to the experimental design and subsequent statistical analysis of in vivo and in vitro comet studies. Teratog Carcinog Mutagen. 19(2), 109-119, 1999.

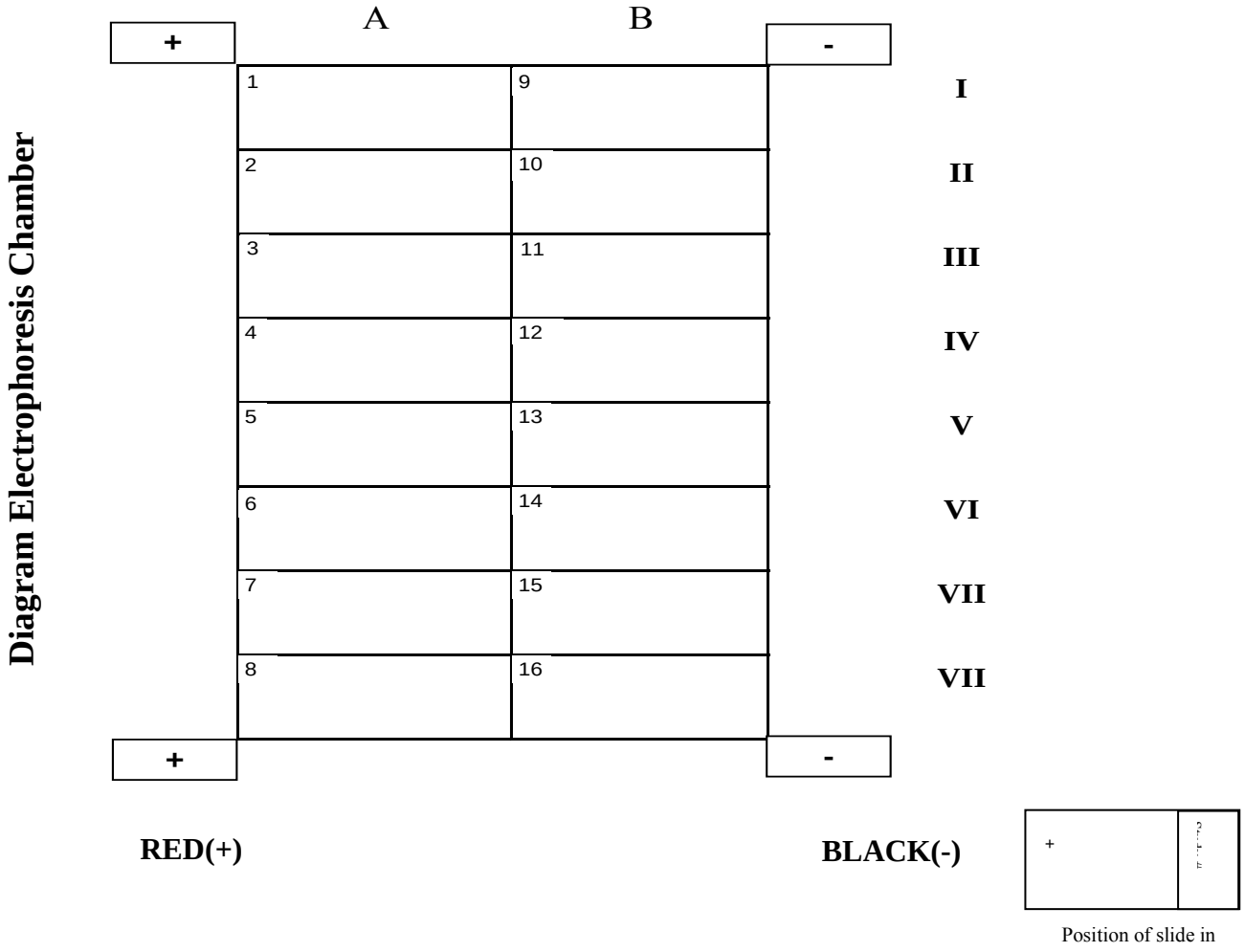
Olive PL, et al., Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cell using the "comet" assay. Radiat. Res., 122, 86-94, 1990.
Tice RR et al., Single cell gel/Comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ. Mol. Mutagen., 35, 206-221, 2000.

Wiklund SJ, E Agurell., Aspects of design and statistical analysis in the Comet assay. Mutagenesis 18(2):167-175, 2003.

Attachment 1:

SLIDES UNWINDING & ELECTROPHORESIS RECORDING SHEET

Electrophoresis Run #				Initials & Date
Approximate alkaline electrophoresis buffer volume in chamber				
Unwinding				
Time	Total	Start	End	
Buffer Temperature				
Electrophoresis				
Running time	Total	Start	End	
Volts				
Milliamperes				
Buffer Temperature				
Thermometer No.				
Electrophoresis chamber No.				
Power supply No.				



Phase4.2 Appendix 2

INTERNATIONAL VALIDATION OF THE *IN VIVO* RODENT ALKALINE COMET ASSAY FOR THE DETECTION OF GENOTOXIC CARCINOGENS
- Study Plan for 2nd Step of 4th Phase Validation Study -

Issued by: the Validation Management Team (VMT)
Date: November 30, 2009

PURPOSE OF THIS DOCUMENT

This document is provided as a supplement to the study protocol to clarify the purpose, schedule, and specific notes of each trial of an international validation study to evaluate the ability of the *in vivo* rodent alkaline Comet assay.

STUDY TITLE

2nd step of 4th phase validation study of international validation of the *in vivo* rodent alkaline Comet assay for the detection of genotoxic carcinogens (abbreviation: 2nd step of 4th phase validation study of *in vivo* Comet assay)

BACKGROUND AND PURPOSE OF THIS STUDY

In the 1st step of the 4th phase validation study, the purpose was to examine the extent of reproducibility and variability of assay results among laboratories using coded test chemicals and the positive control EMS, when experiments were conducted in accordance with the Comet assay protocol-version 14. In brief review of the data, the VMT qualitatively confirmed the reproducibility and variability of assay results among laboratories. Thus the VMT decided to move on the 2nd step of 4th phase validation study with an expanded set of test chemicals in accordance with the Comet assay protocol-version 14.2.

The purpose of the 2nd step is to investigate the predictive capability of the assay against carcinogenicity of test chemicals.

SCHEDULE

November 30, 2009: Agreement of the study plan by the VMT

~December 11, 2009: Delivery of the protocol-version 14.2 and the study plan to testing facilities

~December 31, 2009: Delivery of two or three test chemicals (if two test chemicals are delivered, another will be delivered later)

~January 31, 2010: Delivery of data-spread sheet (Excel file)

January ~ September, 2010: Experimental period (the experiment should be started after acceptance of the study protocol and preparation of appropriate SOPs in each testing facility; the data-spread sheet should be submitted to VMT soon after the data are available)

March 12, 2010: Meeting on Comet international validation study at Salt Lake City, Utah, USA

October 31, 2010: Deadline of data-spread sheet submission to VMT

~January 31, 2011: Data analysis

~March 31, 2011: Finalization of the 2nd step of 4th phase validation study

SPECIFIC NOTES

SUCCESS CRITERIA

To obtain the predictive capability (values of positive sensitivity and negative specificity) of the assay against carcinogenicity of test chemicals (to be discussed in the VMT meeting)

OTHERS

. Dose selection of coded test chemical

The dose levels of each coded test chemical will be decided by each facility. If available, the VMT will provide toxicological information about each coded test chemical, such as the rat LD50, to assist in this process.

Solvent/vehicle

The selection of the solvent/vehicle to use in preparing the dosing formulation for each coded test chemical will be decided by each facility.

Phase 4.2 Appendix 3

Chemical Selection Report

The JaCVAM Initiative International Validation Study of the in Vivo Rodent Alkaline Comet Assay: Selection of Test Chemicals

Author: Takeshi Morita

Status: Draft v. 1.2

Date: August 29, 2012

Summary

The Japanese Center for the Validation of Alternative Methods (JaCVAM) has sponsored a large international prevalidation and validation study of in vivo rat alkaline Comet assay. The main objective of the study was to assess the performance of the assay (i.e., sensitivity and specificity) in correctly identifying carcinogens, as compared with the traditional rat liver UDS assay. Based on existing carcinogenicity and genotoxicity data and chemical class information, 90 chemicals were identified as primary candidates for use in the validation study. In the next process, 46 secondary candidates and following 40 final chemicals were selected from these 90 chemicals based on the sufficiency of carcinogenic and genotoxic data, similarity of chemical class or genotoxic or carcinogenic mode of action (MOA), availability, or price, and ease of handling. These 40 chemicals included 19 genotoxic carcinogens, 6 genotoxic non-carcinogens, 7 non-genotoxic carcinogens and 8 non-genotoxic non-carcinogens. “Genotoxicity” was defined as positive in the Ames test or in one of the standard in vivo genotoxicity tests (primarily the erythrocyte micronucleus assay) in this report. The selected chemicals covered various chemicals classes, MOAs, or genotoxicity profiles. Therefore, they are considered to be suitable for the purpose of the validation study. General principles of chemical selection for validation studies are also discussed.

Introduction

New development and improvement of safety evaluation tests to application for regulatory submission have to be reviewed by experts including regulatory bodies through the validation study described in the OECD Guidance Document 34 (OECD, 2005) for regulatory acceptance. Validation is the process of establishment of reliability and relevance of the tests, and research on validation is a scientific activity for it.

Genotoxicity studies are one of major elements for safety studies. In general, all new chemicals including industrial chemicals or pharmaceuticals, etc. have to be evaluated the potential of genotoxic hazard. The tests for genotoxicity consist of in vitro assays and in vivo assays. The in vivo assays are important in terms of confirmation of in vitro findings and risk evaluation for humans, thus they have more weight than in vitro assays. However, target tissues or organs for in vivo genotoxicity assays are generally hematopoietic cells in bone marrow or blood. Liver is an important target tissue for metabolism of chemicals, especially for evaluation of in vitro positive chemicals with metabolic activation system. However, the application of liver is limited to liver UDS assay as regulatory accepted one which has the OECD test guideline (OECD, 1997). Other tests for possible target of liver will be liver micronucleus test and rodent transgenic mutation test, but the former test is not regulatory accepted yet (Morita et al., 2011). These tests are not recognized as the tests for general routine use because of use of radio isotope, complexity, high cost, or long experimental duration in experimental designs. There are no adequate simple in vivo tests in addition to erythrocyte micronucleus assay. Recently, Comet assay has been developed as a in vivo genotoxicity assay (Burlinson et al., 2007; Kirkland and Speit, 2008; Burlinson, 2012). All tissues or organs in which isolation to single cells is possible are target for the evaluation, and the assay is simple and easy to conduct with low cost. Especially, the usefulness of alternative method of the UDS assay is under consideration as the second in vivo assay following to in vivo micronucleus assay. For regulatory acceptance of Comet assay, it should be recognized as “acceptable method” through the validation study for evaluation of its reliability and relevance, as described above. One of purpose of genotoxicity tests is detection of carcinogens. The test to be validated is preferable to showing high sensitivity (positive results for carcinogens) and high specificity (negative results for non-carcinogens). Therefore, both carcinogens and carcinogens should be used in the validation study for genotoxicity tests. In order to evaluate the Comet assay, the Japanese Center for the Validation of Alternative Methods (JaCVAM) has sponsored a large international prevalidation and validation study of it. Thus, possible suitable test chemicals have been selected for the JaCVAM validation study of in vivo Comet assay.

Methods

Chemical selection strategy

Main purpose of the JaCVAM initiative international validation study of the in vivo rodent alkaline Comet assay is to investigate the predictive capability of the assay against carcinogenicity of test chemicals, and additionally to investigate the alternative capability of the assay against rodent liver UDS assay to detect genotoxic chemicals. To achieve the purpose of the validation study, test chemicals belonging to following four categories based on their genotoxicity and carcinogenicity properties should be selected; 1) genotoxic carcinogens, 2) genotoxic non-carcinogens, 3) non-genotoxic carcinogens, and 4) non-genotoxic non-carcinogens. Therefore, the use of appropriate chemical databases with genotoxic and/or carcinogenic information is necessary. For genotoxic information, data from liver UDS assay and the existing in vivo Comet assay in addition to standard tests including Ames test, in vitro chromosomal aberration test, and in vivo micronucleus test will be considered. Sufficient number of chemicals will be selected as primarily candidate chemicals from the database of potential test chemicals. Then, secondary candidate chemicals will be picked up from the primarily candidates based on several limiting factors such as similarity of chemical class, structure and mode of action, ease of handling, availability, budget, etc. Thus, final chemicals will be selected from the second candidates to adjust chemical numbers to be evaluated in the validation study.

Database of potential test chemicals

Database of potential test chemicals for the validation study should provide appropriate genotoxic and/or carcinogenic information on the chemicals. Several databases were used for primary chemical selection as follows (Table 1):

- ECVAM List

The European Centre for the Validation of Alternative Methods (ECVAM) list is recommended lists of genotoxic and non-genotoxic chemicals for assessment of the performance of new or improved genotoxicity tests (Kirkland et al., 2008). It was discussed by an expert panel in an ECVAM workshop. The list contains 61 chemicals which are Ames-positive or-negative in vivo genotoxins, non-DNA-reactive chemicals (including non-genotoxic carcinogens), or non-carcinogens that are negative or equivocal for genotoxicity in vivo.

- IWGT UDS List

One of main purposes of the validation study is to investigate the alternative capability of the Comet assay against rodent liver UDS assay to detect genotoxic chemicals. Therefore, selection of chemicals with liver UDS data will be important. The International Workshop on Genotoxicity

Testing (IWGT) UDS list is an overview of published findings from in vivo liver UDS tests published up to 1993 (Madle et al., 1994). The list contains 131 chemicals which include 36 positives, 81 negatives, and 14 equivocal chemicals. One hundred and twenty-six chemicals were investigated in male rats, 2 chemicals were examined in female rats, and 20 or 9 chemicals were examined in male or female mice, respectively.

- CSGMT List

The Collaborative Study Group of Micronucleus Test (CSGMT, a working group in the Mammalian Mutagenicity Study group, which is a sub-organization of the Japanese Environmental Mutagen Society) list is the results of a collaborative study on mouse bone marrow and/or peripheral blood micronucleus assay of approximately 100 chemicals classified by the IARC (Groups 1, 2A and 2B) (Morita et al., 1997). The list contains 280 chemicals which include 43, 40, and 197 chemicals in Groups 1 (carcinogenic to humans), 2A (probably carcinogenic to humans) and 2B (possibly carcinogenic to humans), respectively, with in vitro and in vivo genotoxicity data.

- IARC List

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

- CPDB List

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

- NTP List

The National Toxicology Program (NTP) list is a NTP results report by tabular form which contain results, status and publication information on all NTP chemicals (http://www.predictive-toxicology.org/data/ntp/original_ntp_data.txt). It was produced from NTP chemtrack system dated October 8, 2000. The list contains about 2300 chemicals with data on carcinogenicity, genotoxicity and organ systems toxicity.

- EU GHS List

The European Union (EU) has been published the list of harmonised classification and labelling of hazardous substances in Regulation (EC) No 1272/2008 of the European parliament and of the council (EU, 2008). The Globally Harmonised System of Classification and Labelling of Chemicals (GHS) is incorporated as the global harmonisation of criteria for classification. The EU GHS list contains about 4000 chemicals as EU hazardous substances in which many chemicals are classified GHS categories 1A, 1B and 2 for their carcinogenicity or mutagenicity.

Carcinogenicity and genotoxicity

Information on carcinogenicity (carcinogenic, non-carcinogenic) was based on the IARC evaluations. If the candidate chemicals were not included in the IARC list, other database such as CPDB, ECVAM or EU GHS lists were used. Specific animal carcinogens due to by irrelevant mechanism for humans or other species were also considered as carcinogenic. Information on genotoxicity was based on the IARC monographs, ECVAM, IWGT UDS, CSGMT or NTP list. For the chemicals finally selected, precise investigations on in vivo genotoxicity (mainly rats, if not or insufficient, mice were used) were performed by literature survey using PubMed (http://www.ncbi.nlm.nih.gov/pubmed/). Results from existing in vivo Comet assay were also investigated using literatures (e.g., Sasaki et al., 2000; Sekihashi et al., 2002; Kirkland and Speit, 2008). In this report, "genotoxic" was defined as positive in the Ames test or in the standard in vivo genotoxicity tests (mainly erythrocyte micronucleus assay).

Chemical properties

Carcinogens are usually divided into two groups, genotoxic carcinogens and non-genotoxic carcinogens. Variety of chemical classes and carcinogenic or genotoxic mode of action (MOA) are included in genotoxic carcinogens. Thus, chemical properties such as classes, structures and MOA were considered to select chemicals whether Comet assay can apply to wide range of chemicals, or not. These data were based on the reports from IARC monographs (http://monographs.iarc.fr/ENG/Classification/index.php), Kirkland et al. (2008), Morita et al. (1997), Sasaki et al. (2000), or Hazardous Substance Data Bank (HSDB, http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CHEM).

Acute toxicity

It is important to investigate acute toxicity data before the start of experiments. Acute toxicity data will provide good information on the selection of administration doses in the dose-finding study. It affects amount of test chemicals to be provided, resulting in availability in same lot or limitation in budget. As rats will be used for the validation study for the Comet assay, rat oral LD_{50} values were investigated using Registry of Toxic Effects of Chemical Substances

(RTECS, http://csi.micromedex.com/fraMain.asp?Mnu=&Restore=Y) or ChemID Plus (http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CHEM).

Availability

Even if a chemical is considered suitable for the validation study from the scientific point of view, there will be a case that the chemical cannot be selected as test chemical in the international validation study by the following factors: 1) non-marketed product by suitable manufacturer, 2) insufficient amount of chemical in same lot, 3) difficulty in handling of chemicals (gaseous chemical, very small packaging, etc.), 4) restriction of transportation to overseas, and 5) highly expense. Thus, presence or not of marketed products including stocks in major manufacturer of chemical agents, volume of package, and the price were investigated.

Results

Primary candidate chemicals (90 chemicals excluding positive control)

The maximum 43 chemicals was planned to use in the main validation study for Comet assay; the breakdown of the number will be approximately 22 genotoxic carcinogens and 7 chemical each of genotoxic non-carcinogens, non-genotoxic carcinogens, or non-genotoxic non-carcinogens. The number was suggested by the Validation Management Team (VMT) based on the necessity of demonstration of the predictive capability of the assay (sensitivity and specificity) against carcinogenicity and possible number of chemicals tested (i.e., number of participation institutions and duration of the validation study). Thus, 90 chemicals excluding a positive control chemical were selected as primary candidate chemicals (Table 2). The number of chemicals selected was about two times number of each chemical category.

- Genotoxic carcinogens (43 chemicals)

Forty three chemicals including three alternatives were selected as genotoxic carcinogens. Alternatives were ganciclovir for azidothymidine (AZT), and both daunomycin hydrochloride and busulfan for mitomycin C (MMC). AZT or MMC is very important chemical as carcinogenic nucleoside analogue or DNA-cross linker, respectively; but they are expensive. Of 33 chemicals were Ames-positive, remaining 9 chemicals were Ames-negative (including one equivocal), but were positive in in vitro chromosomal aberration (CA) test and rodent in vivo erythrocyte micronucleus test. Majority of chemicals (37/42) were positive in in vitro CA test; one chemical had no data. With respect to the results of liver UDS assay, 13, 12 or 1 chemicals were positive, negative or inconclusive, and remaining 17 chemicals were no data.

- Genotoxic non-carcinogens (13 chemicals)

Thirteen chemicals were selected as genotoxic non-carcinogens. Of 11 chemicals were Ames-positive, remaining 2 chemicals were Ames-negative which were positive in in vitro CA test and rodent in vivo micronucleus test. Majority of chemicals (11/12) were positive in in vitro CA test, one chemical had no data on it. For the results of liver UDS assay, 1, 2 or 1 chemicals were positive, negative or inconclusive, remaining 9 were no data.

- Non-genotoxic carcinogens (19 chemicals)

Nineteen chemicals were selected as non-genotoxic carcinogens. All chemicals were Ames-negative (including one equivocal of methapyrilene hydrochloride). For in vivo erythrocytes micronucleus test, 13, 4 (chloramphenicol, diethylstilbestrol, lead acetate, and trichloroethylene) or

2 (methapyrilene hydrochloride and progesterone) chemicals were negative, inconclusive (presence both positive and negative results), or no data, respectively. Some chemicals (7/19) were positive for in vitro CA test. For the results of liver UDS assay, no chemicals were positive, 8 were negative and remaining 11 were no data. Chloroform, ethanol and methyl carbamate were included as non-genotoxic liver carcinogens to evaluate the effects in liver in the Comet assay.

- Non-genotoxic non-carcinogens (15 chemicals)

Fifteen chemicals were selected as non-genotoxic non-carcinogens. All chemicals were Ames-negative, and all of data available 10 chemicals were also negative for in vivo micronucleus assay. Some chemicals (5/15) were positive for in vitro CA test. For the results of liver UDS assay, no chemicals were positive, 2 were negative, remaining 13 were no data. Sodium chloride was included as a non-genotoxic non-carcinogenic gastrotoxicant to evaluate the effects in stomach in the Comet assay.

- Positive control (1 chemical)

Ethyl methansulfonate, one of genotoxic caricinogen, was used as positive control through the validation study of Comet assay.

Secondary candidate chemicals (46 chemicals excluding positive control)

Forty six chemicals were selected as secondary candidates based on similarity of chemical properties or availability including price among from the 90 primary candidate chemicals (Table 2).

In the main validation study in the Comet assay, five rats per group will be used. Total treatment times by oral dosing will be 4, i.e., one in dose-range finding study and 3 in the main assay. The amount of test chemicals used will depend on the highest dose which will be based on the acute toxicity value. If the highest dose will be 100 mg/kg, minimum amount of test chemical will be 800 mg; 20 mg per rat (100 mg/kg, as 200 g of rat body weight) x 5 (number of rats) x 2 (preparation of dosing solutions) x 4 (number of treatments) = 800 mg. In addition, another amount for selection of vehicle or application of higher dose in dose-range finding study will be needed. Thus, minimum requirement of test chemicals will be 1 g in this case. If the highest dose will be 2000 mg/kg (limit dose), actual consumption is 16 g and minimum requirement will be 20 g. Therefore, chemicals with the price of more than 10,000 JPY per gram were excluded from a view point of budget limitation, in general. Some chemicals were less information on their carcinogenicity and/or genotoxicity, were without suitable supplier, were limitation of stocks with same lot in the supplier, and were unhandy. These chemicals were eliminated from the secondary candidates. Chemicals with duplication of properties, e.g., chemicals class or genotoxic mode of action, were also unselected.

The reasons of unselecting were expensive for 16 chemicals including 2 unhandy ones, similarity for 16 chemicals, less information on carcinogenicity and/or genotoxicity data for 7 chemicals and

no stocks or unhandy for 5 chemicals (Table 2).

Final selection of 40 test chemicals excluding positive control

Forty chemicals were selected as final test chemicals among from the 46 secondary candidate chemicals (Tables 2 and 3). Six deselected chemicals and reasons were as follows: 5 genotoxic carcinogens including acrylamide (due to the use in the phase 2 in the validation study), *N*-methyl-*N*-nitrosourea (due to the use in the phase 3 and phase 4 – step 1 in the validation study), MMC (due to expensive, and the use of busulfan as alternative), daunomycin hydrochloride (due to alternative of MMC) and ganciclovir (due to alternative of AZT), and one non-genotoxic non-carcinogen of D-mannitol (due to the use in the phase 3 and phase 4 – step 1 in the validation study). Though 2,4-diaminotoluene (genotoxic carcinogen) and 2,6-diaminotoluene (genotoxic non-carcinogen) were used in the phase 2 in the validation study, both chemicals were used also in the main validation study to review inter-laboratory reproducibility. Final selection of 40 test chemicals included 19 genotoxic carcinogens, 6 genotoxic non-carcinogens, 7 non-genotoxic carcinogens and 8 non-genotoxic non-carcinogens. They were as follows:

- Genotoxic carcinogens (19 chemicals)

✓ 2-Acetylaminofluorene [53-96-3]

Carcinogenicity: IARC, Not listed; CPDB, positive

2-Acetylaminofluorene was positive for Ames and in vitro CA test and for many kinds of in vivo genotoxicity tests including UDS, MN or rodent transgenic (TG) mutation assay in rats and/or mice. It was positive for Comet assay with mice using one dose level in several organs including liver.

It induced liver tumors in rats and mice, and mammary gland and skin tumors in rats (by CPDB). Metabolic activation is required for its genotoxicity; the first step of it is to N-hydroxy-2-acetylaminofluorene from 2-acetylaminofluorene. It forms C8 bulky adduct on DNA (HSDB, 2003).

✓ Acrylonitrile [107-13-1]

Carcinogenicity: IARC 2B (V71, 1999); CPDB, positive

Acrylonitrile was positive for Ames and in vitro CA test. It was negative for UDS test with rats, MN test with mice and TG mutation test with mice, but positive for MN test with rats treated by iv injection and Comet assay with rat by ip injection. Liver was not evaluated in the TG mutation test.

It induced glial cell tumors of the central nervous system, malignant mammary tumors, Zymbal gland carcinomas, benign and malignant hepatocellular tumors and extrahepatic angiosarcomas in rats. Acrylonitrile is mutagenic, especially after bioactivation by a microsomal

system. Since formation of DNA adducts with acrylonitrile *in vitro* is strongly increased by formation of its epoxide, it is very likely that the genotoxicity of acrylonitrile is mediated primarily by this metabolite (IARC, 1999). Acrylonitrile is metabolized by mammals to cyanide, which then transformed to thiocyanate; more effectively by mice than by rats following oral, ip, and iv administration (HSDB, 2005).

Acrylonitrile vapor is a potent eye, mucous membrane, and skin irritant (HSDB, 2005).

✓ *o*-Anisidine [90-04-0] (*o*-Anisidine hydrochloride [134-29-2])

Carcinogenicity: IARC 2B (V73, 1999); CPDB, positive

o-Anisidine was positive for Ames and in vitro CA test. It was negative for UDS and MN test, but positive for Comet assay in kidney, bladder and lung. It was also positive for TG mutation test in bladder, but not in liver.

o-Anisidine hydrochloride was carcinogenic for rats and mice, inducing transitional cell carcinomas or papillomas of the bladder. Metabolites of o-anisidine led to covalent binding to DNA, and they were consistently more reactive with protein and glutathione than metabolites of *p*-anisidine (IARC, 1999; HSDB, 2005).

o-Anisidine is a skin irritant (HSDB, 2005).

✓ Azidothymidine (AZT) [30516-87-1]

Carcinogenicity: IARC 2B (V76, 2000); CPDB, positive

AZT was positive for in vitro CA test, but not for Ames test. It was also positive for in vivo MN test with rats and mice.

Administration of AZT by gavage induced vaginal squamous-cell carcinomas in mice, and a low incidence of it in rats treated with the highest dose. AZT is incorporated into nuclear and mitochondrial DNA in mammalian cells. It appears to cause mutations primarily by inducing large deletions, consistent with its action as a DNA chain terminator (IARC, 2000).

✓ Benzene [71-43-2]

Carcinogenicity: IARC 1 (V100F, in prep; Supl 7, 1987), CPDB, positive

Benzene was not mutagenic to bacteria and liver in TG mouse. It was clastogenic to mammalian cells in vitro and to rats and mice. Benzene was positive for Comet assay in several organs including liver. It was negative for TG mutation test in liver by inhalation.

Oral doses of benzene induced Zymbal gland carcinomas, mammary carcinomas, and leukemia in rats or mice. Metabolic products in rats are phenol, hydroquinone, catechol, hydroxyhydroquinone, and phenylmercapturic acid (HSDB, 2005).

Benzene is a severe eye and moderate skin irritant (HSDB, 2005).

✓ Busulfan [55-98-1]

Carcinogenicity: IARC 1 (100A, 2011); CPDB, positive

Busulfan was positive for Ames and in vitro CA tests. It induced MN in mice. It was positive for Comet assay in colon, but not liver and stomach.

In a study, intravenous administration of busulfan to mice significantly increased the incidences of thymic and ovarian tumours. Busulfan is a direct-acting bifunctional alkylating agent that binds to cellular macromolecules including DNA, RNA, and proteins. In the liver, it rapidly undergoes both enzymatic and non-enzymatic transformations, primarily through glutathione-mediated processes, to less active, sulfur-containing metabolites (IARC, 2011).

✓ Cadmium chloride [10108-64-2]

Carcinogenicity: IARC 1 (100, 20009); CPDB, positive

Cadmium chloride was not mutagenic to bacteria. It was positive for in vitro CA test and in vivo mouse MN assay. It gave negative results in Comet assay with mice treated with ip injection at single dose level.

Oral administration of cadmium chloride to rats increased the incidence of large granular lymphocytes, leukaemia, prostate tumors, and testis tumors in rats. When ingested, most of the cadmium passes through the gastrointestinal tract without being absorbed. When absorbed, cadmium will bind to metallothionein, forming a cadmium–metallothionein complex that is transferred (via blood) primarily to the liver and the kidney. The genotoxicity of cadmium has to be explained by indirect mechanisms. Frequently discussed mechanisms are related to oxidative stress, the inhibition of DNA-repair systems, effects on cell proliferation, and on tumour-suppressor functions (IARC, 2009).

✓ *p*-Chloroaniline [106-47-8]

Carcinogenicity: IARC 2B (V57, 1993); CPDB, positive

p-Chloroaniline was positive for Ames and in vitro CA tests. It induced MN in mice treated by oral administration for 3 days, but not for single dosing. It gave positive results in Comet assay in various organs in mice.

Oral administration of *p*-chloroaniline induced sarcomas of the spleen and splenic capsule in male rats (IARC, 1993). Whether the mechanism of carcinogenesis is mediated through genotoxic or non-genotoxic events is resolved. p-Chloroaniline is genotoxic in vitro but appears to be dependent on metabolism for its full expression. There is one positive study in vivo (micronucleus test), but this was positive only at a dose level in the range of the LD50 (HSDB, 2005; CICAD, 2003). *p*-Chloroaniline causes methaemoglobinaemia (IARC, 1993).

p-Chloroaniline is slightly irritating to eyes (HSDB, 2005).

✓ Cisplatin [15663-27-1]

Carcinogenicity: IARC 2A (Suppl.7, 1987); CPDB, Not listed

Cisplatin was positive for Ames and in vitro CA tests. It induced MN, DNA damage in colon, lung and brain as Comet, and mutation in liver in mice treated by ip injection.

Cisplatin induced lung adenomas in mice and leukemia in rats by multiple intraperitoneal tretaments (IARC, 1987). Cisplatin can react in a nonenzymatic manner with water in vivo to form monoaquo and diaquo species following dissociation of the chloride groups. These metabolites extensively bind to protein (>90%). Cisplatin can also react with DNA, forming both intrastrand and interstrand cross-links. DNA adducts formed by cisplatin inhibit DNA replication and transcription, and lead to breaks and miscoding (HSDB, 2005).

The substance causes eye irritation or damage (HSDB, 2005).

✓ 2,4-Diaminotoluene [95-80-7]

Carcinogenicity: IARC 2B (Suppl.7, 1987); CPDB, positive

2,4-Diaminotoluene was positive for Ames and in vitro CA tests. It was generally negative for MN assay in mice and rats treated by ip or po. It was positive for Comet assay in several organs including stomach in rats, but not in liver. In mouse TG mutation assay, it induced mutation in liver.

2,4-Diaminotoluene produced hepatocellular carcinomas after oral administration (IARC, 1987). In several rodent species the major urinary metabolite was 2,4-diamino-5-hydroxytoluene; N-acetyl and glucuronide conjugates were also found. 2,4-Diaminotoluene is a mutagenic and hepatocarcinogenic aromatic amine, requiring metabolic activation. It binds to DNA covalently, and increases cell proliferation in livers of animals (HSDB, 2003).

2,4-Diaminotoluene is a skin, eye irritant (HSDB, 2003).

✓ 1,2-Dibromoethane [106-93-4]

Carcinogenicity: IARC 2A (V71, 1999); CPDB, positive

1,2-Dibromoethane was mutagenic to bacteria, and it was clastogenic to mammalian cells in vitro. It was negative for MN assay and TG mutation assay in liver of mice after ip treatment. However, it was positive for Comet assay in multiple organs including liver and stomach, and for liver UDS assay by ip or po treatment.

After oral administration, 1,2-dibromoethane induced squamous cell carcinomas of the forestomach in rats and mice, hepatocellular carcinomas in female rats, hemangiosarcomas in male rats, and aleolar/bronchiolar adenomas in mice (IARC, 1999). Human liver preparations metabolize 1,2-dibromoethane to water soluble and irreversibly protein- and DNA-bound metabolites by both cytochrome p450 (CYP2E1 etc) and glutathione S-transferase enzymes. It binds covalently with DNA in vivo. 1,2-dibromoethane is considered to be a bifunctional alkylating agent which is capable of introducing cross links into biological materials (HSDB, 2005).

It is an irritant to eyes, skin, and mucous membranes (HSDB, 2005).

✓ 1,3-Dichloropropene [542-75-6]

Carcinogenicity: IARC 2B (V71, 1999); CPDB, positive

1,3-Dichloropropene was positive for Ames test, but not in vitro CA test. It was negative for MN and UDS assay with male rodents treated by ip or po. However, 1,3-Dichloropropene induced

micronuclei in the bone marrow of female mice after oral treatment, and DNA damage as Comet in multiple organs of mice after ip treatment.

Technical-grade 1,3-dichloropropene (containing 1.0% epichlorohydrin), when given by gavage, produced tumors of the urinary bladder, lung and forestomach in mice and of the liver and forestomach in rats. The principle metabolic pathway of 1,3-dichloropropene is conjugation with glutathione and elimination as mercapturic acids (IARC, 1999).

It is a severely irritating to skin, eyes, and mucous membranes (HSDB, 2005).

✓ 1,2-Dimethylhydrazine dihydrochloride [306-37-6] (1,2-Dimethylhydrazine [540-73-8])

Carcinogenicity: IARC 2A (V71, 1999); CPDB, positive

1,2-Dimethylhydrazine was positive for Ames and in vitro CA tests. It was also positive for in vivo genotoxicity tests including UDS, MN or Comet in rats after oral treatment.

Whatever the route of administration, 1,2-dimethylhydrazine produced in mice and rats adenomas and adenocarcinomas of the colon. 1,2-Dimethylhydrazine is metabolized by a sequence of oxidation steps, first dehydrogenation to azomethane, N-oxidation of this to azoxymethane and finally a C-oxidation to methylazoxymethanol. This last metabolite decomposes to give the highly reactive methyldiazonium ion (DNA alkylation) to which the carcinogenicity of the compound has been attributed (IARC, 1999).

Contact with the substance may cause irritation to skin, eyes, and mucous membranes (HSDB, 2005).

✓ Hydroquinone [123-31-9]

Carcinogenicity: IARC 3 (V71, 1999); CPDB, positive

Hydroquinone was clasotogenic to mammalian cells in vitro, but not mutagenic to bacteria. It induced micronuclei in the bone marrow of mice after intraperitoneal or oral treatment.

In rats, hydroquinone induced renal tubule adenomas in males. In mice, it induced hepatocellular adenomas in females in one study and in males in another study. Hydroquinone is metabolized mainly to conjugates, but a small percentage may be converted to 1,4-benzoquinone, conjugated with glutathione or form DNA adducts *in vitro* (IARC, 1999).

Hydroquinone is irritating to eyes, skin, and respiratory system (HSDB, 2005).

✓ Methyl methanesulfonate [66-27-3]

Carcinogenicity: IARC 2A (V71, 1999); CPDB, positive

Methyl methanesulfonate was positive for Ames and in vitro CA tests, and for many kinds of in vivo genotoxicity tests including UDS, MN, Comet or TG mutation assay.

Methyl methanesulfonate produced nasal tumors, tumors of the nervous system in rats treated by inhalation exposure and by subcutaneous administration. In mice, it increased the incidence of lung tumours and of lymphomas after oral administration (IARC 71, 1999). Methyl methanesulfonate is one of monofunctional, methylating agents which produce primarily

7-methyl-guanine as an adduct (HSDB, 2008).

✓ *N*-Nitrosodimethylamine [62-75-9]

Carcinogenicity: IARC 2A (Suppl. 7, 1987); CPDB, positive

N-Nitrosodimethylamine was positive for Ames and in vitro CA tests, and for many kinds of in vivo genotoxicity tests including UDS, MN, Comet or TG mutation assay.

N-Nitrosodimethylamine produced liver tumors in male rats treated by sc injection. It induced tumors of nasal cavities and kidneys in rat after inhalation exposures. The metabolic activation of *N*-nitrosodimethylamine by CYP2E1 and following alkylation of DNA are important in its carcinogenic effect (IARC, 1987; HSDB, 2005).

The liquid and vapor may be irritating to the skin or eyes (HSDB, 2005).

✓ 4,4‟-Oxydianiline [101-80-4]

Carcinogenicity: IARC 2B (Suppl.7, 1987); CPDB, positive

4,4‟-Oxydianiline was positive for Ames, in vitro CA, and mouse bone marrow MN tests. It induced DNA damage to liver as Comet in mice, but not as UDS in rats.

4,4‟-Oxydianiline induced hepatocellular carcinomas in rats, adenomas of the Harderian gland in mice, thyroid follicular cell adenomas or carcinomas in rats and mice after feeding exposure (IARC, 1987; HSDB, 2003).

✓ Sodium arsenite [7784-46-5]

Carcinogenicity: IARC 1 (Suppl.7, 1987, 100C, 2009); CPDB, negative

Sodium arsenite was clasotogenic to mammalian cells in vitro, but not mutagenic to bacteria. It induced micronuclei in the bone marrow of mice after intraperitoneal administration.

Sodium arsenite showed some evidence of renal tumor formation in female rats but not in males after oral administration (drinking water). Arsenicals do not react directly with DNA, but cells treated with low concentrations of trivalent arsenicals show increased oxidative DNA damage. As(III) acts as an aneugen by interfering with spindle function and causing micronuclei with centromeres, but at high (toxic) doses, it acts as a clastogen. Oxidative damage to DNA has been shown to cause changes in DNA methylation, suggesting a mechanism by which As(III) may induce this effect (IARC, 2009).

It is irritating to the eyes (HSDB, 2003).

✓ Thioacetamide [62-55-5]

Carcinogenicity: IARC 2B (Suppl.7, 1987); CPDB, positive

Thioacetamide was negative for Ames and in vitro CA tests. However, it was positive for MN test with mice treated by oral administration. In Comet assay, it was positive in stomach, colon and urinary bladder, but not in liver in mice treated by ip injection.

Dietary administration of thioacetamide caused liver cancer (hepatocellular carcinoma) in

mice and rats and tumors of the bile duct in rats. Thioacetamide is oxidized to thioacetamide S-oxide which is not the reactive metabolite responsible for its toxicity, followed by the further metabolism thioacetamide S-oxide to a reactive intermediate which can either bind to liver macromolecules or be further degraded to acetamide and polar products. Thioacetamide is metabolized in vivo to acetamide which is itself carcinogenic (IARC, 1987; HSDB, 2003).

- Genotoxic non-carcinogens (6 chemicals)

✓ 9-Aminoacridine hydrochloride monohydrate [52417-22-8] (9-Aminoacridine hydrochloride [134-50-9], 9-Aminoacridine [90-45-9])

Carcinogenicity: IARC, Not listed; CPDB, Not listed

9-Aminoacridine was positive for Ames and in vitro CA tests. No in vivo genotoxicity data was available.

It is believed to be bacterial frame shift mutagen; it did not induce 6-thioguanidine-resistance in Chinese hamster V79 cells. There is no evidence that 9-aminoacridine is carcinogenic. It is a simple intercalating agent which will not bind covalently to DNA (O"Donovan, 1984).

✓ *p*-Anisidine [104-94-9] (*p*-Anisidine hydrochloride [20265-97-8])

Carcinogenicity: IARC 3 (Suppl.7, 1987); CPDB, negative

p-Anisidine was positive for Ames and in vitro CA tests. No in vivo genotoxicity data was available.

p-Anisidine hydrochloride was not carcinogenic in male and female mice and female rats treated by the feed. Equivocal evidence was obtained in male rats (CPDB, HSDB, 2005).

✓ 2,6-Diaminotoluene [823-40-5] (2,6-Diaminotoluene dihydrochloride [15481-70-6])

Carcinogenicity: IARC, Not listed; CPDB, negative

2,6-Diaminotoluene was positive for Ames and in vitro CA tests. However, results from in vivo genotoxicity studies were conflict. Relatively high dose of 2,6-diaminotoluene induced UDS, MN, and Comet in rats after oral administration. The chemical was also positive for MN test with mice after ip treatment. TG mutation assay was negative in the liver of mice treated by diet.

2,6-Diaminotoluene dihydrochloride was not carcinogenic for male and female rats or for male and female mice after 2-year feeding (HSDB, 2003). The carcinogen 2,4-diaminotoluene produced a dose dependent increase in cell proliferation of approximately 10 – 20 % in livers of animals, whereas the noncarcinogen 2,6-diaminotoluene produced no increase in cell turnover compared to vehicle control. These results indicate a positive correlation between increased cell proliferation and hepatocarcinogenesis induced by these two isomers of diaminetoluene (HSDB, 2003).

✓ 5-Fluorouracil [51-21-8]

Carcinogenicity: IARC 3 (Suppl.7, 1987); CPDB, positive

5-Fluorouracil was positive for in vitro CA test, but not for Ames test. It induced MN in rats after ip or po dosing. It was negative for Comet assay with mice treated by ip injection.

Though CPDB described that 5-fluorouracil induced tumor in lung or hematopoietic system in mice, it is believed that there is no evidence of carcinogenicity. Long-term studies in animals to determine the carcinogenic potential of 5-fluorouracil have not been performed; however, no evidence of carcinogenicity was observed in several animal studies following oral or iv administration of the drug for up to 1 year (IARC, 1987; HSDB, 2007) . 5-Fluorouracil is an antimetabolite of the pyrimidine analog type. It is considered to be cell cycle-specific for the S phase of cell division. Activity results from its conversion to an active metabolite in the tissues, and includes inhibition of DNA and RNA synthesis (HSDB, 2007).

✓ 8-Hydroquinoline [148-24-3]

Carcinogenicity: IARC 3 (Suppl.7, 1987); CPDB, negative

8-Hydroquinoline was positive for Ames and in vitro CA tests. However, in vivo genotoxicity tests including UDS, MN or Comet assay gave negative results in rats or mice.

8-Hydroxyquinoline, given at 1500-3000 ppm in the feed of male and female rats and mice for 103 weeks, showed no evidence of carcinogenicity. On the other hand, structurally related chemical of quinolone is a specific and potent carcinogen to the rat and mouse liver. 8-Hydroxyquinoline was metabolized to glucuronide and sulfate conjugates after iv administration (HSDB, 2002).

✓ *p*-Phenylenediamine dihydrochloride [624-18-0] (*p*-Phenylenediamine [106-50-3])

Carcinogenicity: IARC 3 (Suppl.7, 1987); CPDB, negative

p-Phenylenediamine was positive for Ames and in vitro CA tests. However, it gave negative results in MN or UDS assay with rodents.

There was no convincing evidence that dietary administration of p-phenylenediamine dihydrochloride was carcinogenic in rats or mice (HSDB, 2002).

● Non-genotoxic carcinogens (7 chemicals)

✓ Chloroform [67-66-3]

Carcinogenicity: IARC 2B (V73, 1999); CPDB, positive

Chloroform was negative for standard in vitro and in vivo genotoxicity tests including Ames, CA, UDS, MN, Comet and TG mutation tests. It induced micronuclei in kidney cells of rats co-treated with folic acid.

Chloroform induced kidney epithelial tumors in male rats and male mice treated by oral administration (gavage or drinking water), and hepatocellular carcinomas in mice by gavage. The

metabolism of chloroform is via CYP2E1-mediated oxidation to phosgene. The rate of metabolism is greatest in liver, kidney cortex, and nasal mucosa (HSDB, 2009). Chloroform has been shown to induce cytolethality and regenerative cell proliferation in the target organs for cancer (IARC, 1999).

It is a skin and eye irritant (HSDB, 2009).

✓ Diethanolamine [111-42-2]

Carcinogenicity: IARC 2B (V101, in prep); CPDB, Not listed

Diethanolamine was negative for Ames and in vitro CA tests. It did not induce micronucleated normochromatic erythrocytes in blood of mice treated by skin application for 90 days.

There was no evidence of carcinogenic activity of diethanolamine in rats after 2-year dermal studies. On the other hand, there was clear evidence of it in mice based on increasing incidences of liver neoplasms and renal tubule neoplasms (HSDB, 2005). In mice, diethanolamine alters choline homeostasis in a manner resembling choline deficiency. Diethanolamine-induced choline deficiency thus provides a mechanism for the tumorigenesis noted in mice but not in rats. (IARC, 2000)

It is a slight irritation of skin and mucous membranes (HSDB, 2005).

✓ Di(2-ethylhexyl)phthalate [117-81-7]

Carcinogenicity: IARC 2B (V101, in prep): CPDB, positive

Di(2-ethylhexyl)phthalate was negative for standard in vitro and in vivo genotoxicity tests including Ames, CA, UDS, MN, Comet and TG mutation tests.

Di(2-ethylhexyl)phthalate induced hepatocellular adenomas and carcinomas in mice and rats treated by oral (feed) exposure. Additional studies found an increased incidence of pancreatic acinar-cell adenomas in male rats, and an increased incidence of Leydig-cell tumors in rats. There was argument that it caused liver tumors in rats and mice by a non-DNA-reactive mechanism involving peroxisome proliferation, which was considered not relevant to humans previously. Since then, additional mechanistic information has become available; the human relevance of the molecular events leading to DEHP-induced cancer in several target tissues (eg, liver and testis) in rats or mice could not be ruled out (IARC, in prep).

✓ Ethanol [64-17-5]

Carcinogenicity: IARC 1 (V96, 2010; V100E, in prep); CPDB, positive

Ethanol was negative for Ames and in vitro CA tests. It did not induce micronuclei in mice treated by drinking water for 7 weeks.

Ethanol induced hepatocellular adenoma or carcinoma in male mice, not but in female mice treated orally in the drinking water (IARC, 2010). In male rats, tumors were induced in adrenal grand, liver, pancreas, or pituitary gland. Ethanol is metabolized by alcohol dehydrogenase to acetaldehyde that is a genotoxic compound that is de toxified by aldehyde dehydrogenases. However, the mechanisms of the induction of cancer by consumption of alcoholic beverages and

more specifically ethanol are not entirely clear, and are certainly complex (IARC, 2010).

It is an eye irritant (HSDB, 2006).

✓ Methyl carbamate [598-55-0]

Carcinogenicity: IARC 3 (Suppl.7, 1987); CPDB, positive

Methyl carbamate was negative for Ames and in vitro CA tests. It did not induce micronuclei in mice after ip treatment.

Methyl carbamate induced hepatocellular neoplastic nodules and hepatocellular carcinomas in male and female rats in 2-year gavage studies. It showed no evidence of carcinogenic activity for male and female mice (HSDB, 2003). Liver tumors in rats will be due to inflammation and hyperplasia resulting from bioaccumulation (poor clearance) (Kirkland et al., 2008).

✓ *o*-Phenylphenol sodium salt [132-27-4] (*o*-Phenylphenol [90-43-7])

Carcinogenicity: IARC 2B for sodium salt (IARC 73, 1999) and IARC 3 for free base (IARC 73, 1999); CPDB, positive

o-Phenylphenol was positive for in vitro CA test, but not for Ames test. It did not induce CA or MN in bone marrow cells of rodents. However, it was positive for MN assay with urinaly bladder of rats. Comet assay gave conflict results with rats (one positive and one negative) or mice (one positive) after oral treatment.

The carcinogenicity of *o*-phenylphenol or *o*-phenylphenol sodium salt toward the urinary bladder was demonstrated when rats were chronically fed concentrations of 0.5%-4% in their diet. Other species tested so far did not develop tumors. *o*-Phenylphenol produces cytotoxicity and proliferation of the urothelium without formation of urinary solids. The paucity of unconjugated metabolites and the lack of DNA adducts suggests that *o*-phenylphenol is acting as a bladder carcinogen in male rats by inducing cytotoxicity and hyperplasia without it or its metabolites directly binding to DNA (HSDB, 2003).

It is irritating to skin and the eyes (HSDB, 2003).

✓ Saccharin [81-07-2] (Saccharin sodium salt [128-44-9])

Carcinogenicity: IARC 3 (V73, 1999); CPDB, positive for sodium salt and negative for free base

Saccharin was negative for Ames and in vitro CA tests. It did not induce CA in bone marrow cells of mice or mutation in liver of TG rats treated by po (gavage or diet). However, it was positive for Comet assay with mice (stomach and colon) after po treatment.

Sodium saccharin produced urothelial bladder tumors in rats by a non-DNA reactive mechanism that involves the formation of a urinary calcium phosphate containing precipitate, cytotoxicity and enhanced cell proliferation. It is not carcinogenic to other species (HSDB, 2003).

- Non-genotoxic, non-carcinogens (8 chemicals)

✓ Ampicillin trihydrate [7177-48-2] (Ampicillin [69-53-4])

Carcinogenicity: IARC 3 for free base (V50, 1990); CPDB, negative

Ampicillin was negative for Ames and in vitro CA tests. It did not induce MN in rats. There was equivocal evidence of carcinogenicity of ampicillin trihydrate for male rats as shown by increased incidences of pheochromocytomas of the adrenal medulla and marginally increased incidences of mononuclear cell leukemia in 2-year gavage studies. There was no evidence of carcinogenicity for female rats or for male or female mice (HSDB, 2003).

✓ *o*-Anthranilic acid [118-92-3]

Carcinogenicity: IARC 3 (V16, 1978, Suppl. 7, 1987); CPDB, negative

o-Anthranilic acid was positive for in vitro CA test, but not for Ames test. It did not induce MN in bone marrow cells of mice.

Anthranilic acid was not carcinogenic for either rats or mice under the conditions of a bioassay in which animals were treated by feed (HSDB, 2008).

The substance irritates the eyes (HSDB, 2008).

✓ *t*-Butylhydroquinone [1948-33-0]

Carcinogenicity: IARC, Not listed; CPDB, negative

t-Butylhydroquinone was positive for in vitro CA test, but not for Ames test. It did not induce MN in bone marrow cells of mice.

There was no evidence of carcinogenic activity of t-butylhydroquinone in rats or mice under the conditions of 2-year feed study (HSDB, 2003).

It is a weak dermal irritant (HSDB, 2003).

✓ Ethioamide [536-33-4]

Carcinogenicity: IARC 3 (V13, 1977, Suppl.7, 1987); CPDB, positive

Ethioamide was negative for Ames test, but weak positive for in vitro CA test at 5-8 mM with precipitate (Kirkland et al, 2008). There was no in vivo genotoxicity data.

Ethionamide was not carcinogenic to rats, but induced thyroid tumors in female mice (Kirkland et al, 2008). However, a report mentioned that no lesions of the thyroid were found in the dosed rats or mice at an incidence above that in the matched controls, and concluded that under the conditions of this feeding bioassay, ethionamide was not carcinogenic in either rats or mice (NCI, 1987).

✓ Isobutylaldehyde [78-84-2]

Carcinogenicity: IARC, Not listed; CPDB, negative

Isobutylaldehyde was negative for Ames test or in vitro CA test with S9, but positive for in vitro CA test without S9, which might be due to oxidative mechanism (Kirkland et al, 2008). It did not induce MN in mice or rats.

There was no evidence of carcinogenic activity of isobutyraldehyde in rats or mice in 2-year inhalation (6 hr/day, 5 days/week) studies (HSDB, 2003).

It is irritating to skin and eyes (HSDB, 2003).

✓ D,L-Menthol [15356-70-4]

Carcinogenicity: IARC, Not listed; CPDB, negative

D,L-Menthol was negative for Ames test, but positive for in vitro CA test with toxicity in the absence of S9 (Kirkland et al, 2008). It was negative for MN or Comet assay with mice treated by ip or oral administration, respectively.

D,L-Menthol was not carcinogenic for either rats or mice in 2-year feeding bioassay (HSDB, 2003).

It is moderate irritant to mucous membranes on inhalation (HSDB, 2003).

✓ Sodium chloride [7647-14-5]

Carcinogenicity: IARC, Not listed; CPDB, negative

Sodium chloride was negative for Ames or in vitro CA test. It was also negative for stomach UDS assay with rat after po dosing, or for bone marrow MN assay with mice treated by ip administration.

Sodium chloride did not induce tumors in male rats or in male and female mice. Sodium chloride given alone had no apparent carcinogenicity but it promoted tumors in the glandular stomach and forestomach (HSDB, 2007)..

✓ Trisodium ethylenediamine tetraacetic acid monohydrate [10378-22-0] (Ethylenediamine tetraacetic acid (EDTA) [60-00-4], Trisodium EDTA trihydrate [150-38-9], Disodium EDTA dehydrate [6381-92-6])

Carcinogenicity: IARC, Not listed; CPDB, negative for EDTA trihydrate [150-38-9]

Ethylenediamine tetraacetic acid (EDTA) was negative for Ames or in vitro CA test. It was also negative for mouse bone marrow MN assay or mouse Comet assay. There was positive finding in MN test in mice after ip dosing at relatively low dose levels, but the results is questionable (EU, 2004).

Although a variety of tumors occurred among test and control animals of both rats and mice in 2-year feeding bioassay with trisodium EDTA trihydrate, no tumors were related to treatment (HSDB, 2004).

Discussion

Test chemical selection for validation study has a major impact on the success or failure of the study. Suitable number of chemicals with specific properties should be selected to achieve the purpose of the validation study.

In the beginning, 43 as maximum number of chemicals to be used was derived from VMT based on the necessity of demonstration of the predictive capability of the assay (sensitivity and specificity) against carcinogenicity, in the Comet validation study. Several databases were used for the selection of primary candidate chemicals (Table 1). Then, 90 chemicals excluding positive control were selected as primary candidates. They included genotoxic carcinogens, genotoxic non-carcinogens, non-genotoxic carcinogens, or non-genotoxic non-carcinogens. In the next, 46 or 40 chemicals were selected as secondary candidates or final chemicals, respectively, based on similarity of chemical properties, availability, or necessity for review of inter-laboratory reproducibility among from primary or secondary candidate (Table 2). The final chemicals selected were based on the consideration of carcinogenicity, in vitro and in vivo standard genotoxicity assay results including existing UDS and Comet assay, chemical class, genotoxic MOA, or availability (Tables 2 and 3). Chemical classes of them were summarized in Table 4. Nineteen genotoxic carcinogens were selected from wide range of classes including 7 aromatic compounds including aromatic amines, 3 aliphatic compounds including aliphatic halides, 1 heterocyclic compound, 3 metal compounds, 1 hydrazine, 2 sulfonates and 1 amide. Genotoxic or carcinogenic MOA of them is DNA alkylation, DNA cross-linking, adduct formation including bulky one, nucleoside analogue, aneugenic effect, possible DNA repair inhibition or cytotoxic effect. Chemicals belong to other categories (i.e., genotoxic non-carcinogens, non-genotoxic carcinogens, and non-genotoxic non-carcinogens) were mainly aromatic compounds, aliphatic compounds, or heterocyclic compounds (Table 4). Carcinogenic MOA of the non-genotoxic carcinogens selected includes hormonal effect, cytotoxicity, choline deficiency, peroxisome proliferation or inflammation. With respect to UDS data in the 40 final chemicals, 6, 8 or 1 chemical showed positive, negative, or inconclusive, respectively. Remaining 25 chemicals were not tested on UDS. Thus, final 40 chemicals were consisted of various chemicals classes, MOA, or genotoxicity profiles. Therefore, these are considered to be suitable for purpose of the validation study, i.e., predictive capability of the assay against carcinogenicity and alternative capability of the assay against rodent liver UDS assay.

The process of chemical selection used in the Comet validation study will be applicable for other validation study. It consists of derivation of maximum number of chemicals to be tested, selection of databases used, collection of candidate chemicals, scientific refinements and practical refinements (Table 5). These general principals of chemical selection will be useful for validation study.

References

Burlinson, B., R. R. Tice, G. Speit, E. Agurell, S. Y. Brendler-Schwaab, A. R. Collins, P. Escobar, M. Honma, T. S. Kumaravel, M. Nakajima, Y. F. Sasaki, V. Thybaud, Y. Uno, M. Vasquez, A. Hartmann, Fourth International Workgroup on Genotoxicity Testing: result of the in vivo comet assay workgroup, Mutat. Res. 627 (2007), 31-35.

Burlinsone, B. In vitro and in vivo comet assays, Methods Mol. Biol., 817, 143-163, 2012.

European Union (EU), Edetic acid (EDTA), European Union Risk Assessment Report, volume 49, 2004. < http://esis.jrc.ec.europa.eu/index.php?PGM=ora>

EU, Regulation (EC) No 1272/2008 of the European parliament and of the council of 16 December 2008 on classification, labelling and packaging of substances and mixtures, amending and repealing Directives 67/548/EEC and 1999/45/EC, and amending Regulation (EC) No 1907/2006, Official Journal of the European Union, L 353/1, 31.12.2008. < http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2008:353:0001:1355:en:PDF> (access December 2009)

Hazardous Substance Data Bank (HSDB), 2-Acetylaminofluoerene, HSDB Number 4077, Last revision: August 29, 2003. <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CHEM>

HSDB, Acrylonitrile, HSDB Number 176, Last revision: June 24, 2005. <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CHEM>

HSDB, *o*-Anisidine, HSDB Number 2073, Last revision: June 23, 2005. <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CHEM>

HSDB, Benzene, HSDB Number 35, Last revision: June 24, 2005. <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CHEM>

HSDB, 4-Chloroaniline, HSDB Number 2047, Last revision: December 13, 2005. <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CHEM>

HSDB, cis-Diaminedichloroplatinum, HSDB Number 3939, Last revision: June 23, 2005. <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CHEM>

HSDB, Toluene-2,4-diamine, HSDB Number 2849, Last revision: August 29, 2003. <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CHEM>

HSDB, Ethylene dibromide, HSDB Number 536, Last revision: June 24, 2005. <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CHEM>

HSDB, 1,3-Dichloropropene, HSDB Number 1109, Last revision: June 24, 2005. <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CHEM>

HSDB, 1,2-Dimethylhydrazine, HSDB Number 4039, Last revision: January 31, 2005. <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CHEM>

HSDB, Hydroquinone, HSDB Number 577, Last revision: June 24, 2005. <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CHEM>

HSDB, Methyl methanesulfonate, HSDB Number 5103, Last revision: April 14, 2008. <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CHEM>

HSDB, N-Nitrosodimethylamine, HSDB Number 1667, Last revision: June 23, 2005. <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CHEM>

HSDB, Bis(4-aminophenyl) ether, HSDB Number 1316, Last revision: August 29, 2003. <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CHEM>

HSDB, Sodium arsenite, HSDB Number 693, Last revision: March 5, 2003. <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CHEM>

HSDB, Thioacetamide, HSDB Number 1318, Last revision: August 29, 2003. <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CHEM>

HSDB, *p*-Anisidine, HSDB Number 1603, Last revision: June 24, 2005. <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CHEM>

HSDB, 2,6-Diaminotoluene, HSDB Number 4131, Last revision: February 14, 2003. <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CHEM>

HSDB, Fluorouracil, HSDB Number 3228, Last revision: October 11, 2007. <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CHEM>

HSDB, 1,4-Diaminobenzene dihydrochloride, HSDB Number 5436, Last revision: August 6, 2002. <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CHEM>

HSDB, Chloroform, HSDB Number 56, Last revision: May 5, 2009. <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CHEM>

HSDB, Ethanol, HSDB Number 82, Last revision: April 14, 2006. <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CHEM>

HSDB, Methyl carbamate, HSDB Number 2587, Last revision: February 14, 2003. <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CHEM>

HSDB, *o*-Phenylphenol, HSDB Number 1753, Last revision: April 2, 2003. <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CHEM>

HSDB, Saccharin, HSDB Number 669, Last revision: August 29, 2003. <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CHEM>

HSDB, Ampicillin, HSDB Number 3009, Last revision: February 14, 2003. <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CHEM>

HSDB, Anthranilic acid, HSDB Number 1321, Last revision: April 22, 2008. <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CHEM>

HSDB, *t*-Butylhydroquinone, HSDB Number 838, Last revision: March 5, 2003. <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CHEM>

HSDB, Isobutylaldehyde, HSDB Number 614, Last revision: January 24, 2003. <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CHEM>

HSDB, Menthol, HSDB Number 593, Last revision: March 5, 2003. <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CHEM>

HSDB, Sodium chloride, HSDB Number 6368, Last revision: June 6, 2007. <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CHEM>

HSDB, Ethylenediamine tetraacetic acid, HSDB Number 809, Last revision: September 10, 2004. <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CHEM>

HSDB, Diethanolamine, HSDB Number 924, Last revision: August 23, 2005. <http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?CHEM>

International Agency on Research on Cancer (IARC), Re-Evaluation of Some Organic Chemicals, Hydrazine and Hydrogen Peroxide, IARC monographs on the evaluation of carcinogenic risks to humans, volume 71, 1999. <http://monographs.iarc.fr/>

IARC, Some chemicals that cause tumours of the kidney or urinary bladder in rodents and some other substances, IARC monographs on the evaluation of carcinogenic risks to humans, volume 73, 1999. <http://monographs.iarc.fr/>

IARC, Some antiviral and antineoplastic drugs, and other pharmaceutical agents, IARC monographs on the evaluation of carcinogenic risks to humans, volume 76, 2000. <http://monographs.iarc.fr/>

IARC, A review of human carcinogens, Part A: Pharmaceuticals, IARC monographs on the evaluation of carcinogenic risks to humans, volume 100, 2009. <http://monographs.iarc.fr/>

IARC, Occupational exposures of hairdressers and barbers and personal user of hair colourants, some hair dyes, cosmetic colourants, industrial dyestuffs and aromatic amines, volume 57, 1993. <http://monographs.iarc.fr/>

IARC, Overall Evaluations of Carcinogenicity: An Updating of IARC Monographs Volumes 1 to 42, Supplement 7, 1987. <http://monographs.iarc.fr/>

IARC, A review of human carcinogens, Part C: Arsenic, Metals, Fibres, and Dusts, IARC monographs on the evaluation of carcinogenic risks to humans, volume 100, 2009. <http://monographs.iarc.fr/>

IARC, Some industrial chemicals, IARC monographs on the evaluation of carcinogenic risks to humans, volume 77, 2000. <http://monographs.iarc.fr/>

IARC, Some chemicals in industrial and consumer products, some food contaminants and flavourings, and water chlorination by-products, IARC monographs on the evaluation of carcinogenic risks to humans, volume 101. in preparation. <http://monographs.iarc.fr/>

IARC, Alcohol consumption and ethyl carbamate, IARC monographs on the evaluation of carcinogenic risks to humans, volume 96, 2010. <http://monographs.iarc.fr/>

International Programme on Chemical Safety (IPCS), 4-Chloroaniline, Concise International Chemical Assessment Document, volume 48, 2003.

<http://www.who.int/ipcs/publications/cicad/en/>

Kirkland, D. and G. Speit, Evaluation of the ability of a battery of three *in vitro* genotoxicity tests to discriminate rodent carcinogens and non-carcinogens III. Appropriate follow-up testing *in vivo*, Mutat. Res. 654 (2008), 114-132.

Kirkland, D., Kasper, P., Muller, L., Corvi, R., Speit, G., Recommended lists of genotoxic and non-genotoxic chemicals for assessment of the performance of new or improved genotoxicity tests: A follow-up to an ECVAM workshop, Mutat. Res., 653, 99-108, 2008.

Madle, S., S.W. Dean, U. Andae, G. Brambilla, B. Burlinson, D.J. Doolittle, C. Furihata, T. Hertner, C.A. McQueen, H. Mori, Recommendations for the performance of UDS tests in vitro and in vivo, Mutat Res., 312, 263-285, 1994.

Morita, T., N. Asano, T. Awogi, Y.F. Sasaki, S. Sato, H. Shimada, S. Sutou, T. Suzuki, A. Wakata, T. Sofuni and M. Hayashi: Evaluation of the rodent micronucleus assay in the screening of IARC carcinogens (Groups 1, 2A, and 2B), The summary report of the 6th collaboration study by CSGMT/JEMS･MMS, Mutation Res., 389, 3-122, 1997 [Erratum, Mutation res., 391, 259-267, 1997].

Morita, T. MacGregor, J.T. and M. Hayashi: Micronucleus assays in rodent tissues other than bone marrow, Mutagenesis, 26, 223-230, 2011.

National Cancer Institute (NCI), Bioassay of ethinamide for possible carcinogenicity, CAS No. 536-33-4. Technical Report Series, No. 46, NCI-CG-TR-46, 1978. < http://ntp.niehs.nih.gov/ntp/htdocs/LT_rpts/tr046.pdf> (access March 2010)

O'Donovan, MR., Mutagenicity of some intercalating agents in Chienese hamster V79 cells, Mutat. Res., 137, 117-122, 1984.

Organisation for Economic Co-operation and Development (OECD), OECD guideline for the testing of chemicals, No. 486, Unscheduled DNA synthesis (UDS) test with mammalian liver cells in vivo, adopted 21 July1997.

OECD, Series on testing and assessment Number 34, Guidance document on the validation and international acceptance of new or updated test methods for hazard assessment, ENJ/JM/MONO, 14 2005. <http://appli1.oecd.org/olis/2005doc.nsf/linkto/env-jm-mono(2005)14> (access December 2009)

Sasaki, Yu F., K. Sekihashi, F. Izumiyama, E. Nishidate, A. Saga, K. Ishida, S. Tsuda, The comet assay with multiple mouse organs: Comparison of comet assay results and carcinogenicity with 208 chemicals selected from the IARC monographs and U.S. NTP carcinogenicity database, Crit. Rev. Toxicol., 30, 629-799, 2000.

Sekihashi, K., A. Yamamoto, Y. Matsumura, S. Ueno, M. Watanabe-Akanuma, F. Kassie, S. Knasmuller, S. Tsuda, Y.F. Sasaki, Comparative investigation of multiple organs of mice and rats in the comet assay, Mutat Res., 517, 53-74, 2002.

From Table 3

1. Madle et al, Mutat. Res., 312, 263-285, 1994.
2. Mirsalis et al, Environ. Mutagen., 4, 553-562, 1982.
3. Wakata et al., Environ. Mol. Mutagen., 32, 84-100, 1998.
4. Sasaki, Crit Rev Toxicol. 30, 629-799, 2000.
5. Sekihashi et al, Mutat. Res., 493, 39-54, 2001.
6. Butterworth et al, Environ. Mol. Mutagen., 20, 148-155, 1992.
7. Morita et al, Mutat. Res., 389, 1-122, 1997.
8. Sekihashi et al, Mutat. Res., 517, 53-74, 2002.
9. Ashby et al., Mutat. Res., 250, 115-133, 1991.
10. Lambert et al, Mutat. Res., 590, 1-280, 2005.
11. Ashby et al., Carcinogenesis, 15, 2291-2296, 1994.
12. Kirkland et al., Mutat. Res., 653, 99-108, 2008.
13. PIPCS/WHO, CICAD 48, 2003.
14. NTP Toxicity Report 43, 1998.
15. BUA Report 153 (1995)
16. Sasaki et al, Mutat. Res., 440, 1-18, 1999.
17. Tsuda et al, Mutat. Res., 467, 83-98, 2000.
18. George and Westmoreland, Carcinogenesis, 12, 2233-2237, 1991.
19. Suter et al, Environ. Mol. Mutagen., 28, 354-362, 1996.
20. Working et al, Carcinogenesis, 7, 467-472, 1986.
21. Sasaki et al, Mutat. Res., 419, 13-20, 1998.
22. Ghiha et al, Toxicol. Appl. Pharmacol., 120, 120-125, 1993.

23. Takasawa et al, Mutat. Res., 698, 24-29, 2010.
24. Mavournin et al, Mutat. Res., 239, 29-80, 1990.
25. Watanabe et al, Mutat. Res., 97, 43-48, 1982.
26. Mirsalis et al., Environ. Mol. Mutagen., 14, 155-164, 1989.
27. Shelby et al, Environ. Mol. Mutagen., 21, 160-179, 1993.
28. Phillips et al, Environ. Mol. Mutagen., 18, 168-183, 1991.
29. Dertinger et al, Mutat. Res., 368, 301-307, 1996.
30. Celik et al, Toxicol. Mech. Methods, 19, 135-140, 2009.
31. Fahmy and Aly, J. Appl. Toxicol., 20, 231-238, 2000.
32. Adler and Kliesch, Mutat. Res., 234, 115-123, 1990.
33. IPCS/WHO, EHC 157, 1994.
34. Ciranni et al, Mutat. Res., 209, 23-28, 1988.
35. Twinwell et al, Environ. Health Perspect., 95, 205-210, 1991.
36. Allavena et al, Teratogen. Carcinogen. Mutagen., 12, 31-41, 1992.
37. Toyoda-Hokaiwado et al, Toxicol. Science, 114, 71-78, 2010.
38. Suzuki et al, Mutagenesis, 24, 9-16, 2009.
39. Sasaki et al, Mutata. Res., 391, 215-230, 1997.
40. Ashby et al, Environ. Mol. Mutagen., 14, 221-228, 1989.
41. Soler-Niedziela et al, Mutat. Res., 259, 43-48, 1991.
42. IARC, v73, 1999.
43. Gock et al, Mutat. Res., 90, 91-109, 1981.
44. Robbiano et al, Mutat. Res., 413, 1-6, 1998.
45. Gunz et al, Environ. Mol. Mutagen., 21, 209-211, 1993.
46. Phillips and Jenkinson, Mutagenesis, 16, 91-101, 2001.
47. Shelby and Tice, Mutat. Res., 260, 311, 1991.
48. Brusick, Environ. Mol. Mutagen., 45, 460-481, 2005.
49. Brendler-Schwaab et al, Mutagenesis, 20, 245-254, 2005.
50. Yoshida and Hiraga, Ann Rep Tokyo Metr Res Lab PH, 33, 489-491, 1982.
51. Shirasu et al, Mutat. Res., 54, 227, 1978 (abstract)
52. Balakrishnan and Eastmond, Fd Chem Toxicol., 44, 1340-1347, 2006.
53. Balakrishan et al, Mutagenesis, 17, 89-93, 2002.
54. Bomhard et al, Crit. Rev. Toxicol., 32, 551-625, 2002.
55. Sasaki et al, Mutat. Res., 519, 103-119, 2002.
56. Ashby, Fd. Chem. Toxicol., 23, 507-519, 1985.
57. Chieli et al, Mutat. Res, 192, 141-143, 1987.
58. NTP Database, http://www.ntp-server.niehs.nih.gov
59. Stemp et al, Mutagenesis, 4, 439-445, 1989.
60. McFee et al, Environ. Mol. Mutagen., 14, 207-220, 1989.
61. Ohsawa et al, Mutat. Res., 287, 307-319, 1993.

62. IARC, V76, 2000
63. Rothfuss et al., Mutat. Res., 702, 40-69, 2010.
64. Hayward et al., Carcinogenesis, 16, 2429-2433, 1995
65. Shephard et al., Mutat. Res., 302, 91-96, 1993.
66. Louro et al., Environ. Mol. Mutagen., 40, 283-291, 2002.
67. Hachiya and Motohashi, Ind. Health, 38, 213-220, 2000.
68. Mirkova, Mutat. Res., 352, 23-30, 1996.
69. Witt et al, Environ. Mol. Mutagen., 36, 163-194, 2000.
70. Leonard and Leonard, J. Environ. Path. Toxicol. 2, 1047, 1979.
71. Tates et al., Mutat. Res., 79, 285-288, 1980.
72. EU Risk Assessment Report, V49, 2004.
73. NTP Results Report, 2000.
(http://www.predictive-toxicology.org/data/ntp/original_ntp_data.txt)
74. Ishidate et al., Mutat Res., 195, 151-213, 1988
75. IARC, v96, 2010.
76. Ghia et al., Toxicol. Apple. Pharmacol., 120, 120-125, 1983.
77. Kevekordes et al., Toxicol. Lett. 89, 35-42, 1996.
78. IARC, V16, 1978.

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

Database	Number of chemicals listed	Note	Ref.
ECVAM List	61	Recommended lists of genotoxic and non-genotoxic chemicals for assessment of the performance of new or improved genotoxicity tests	Kirkland et al., 2008
IWGT UDS List	131	Overview of published findings from in vivo liver UDS tests	Madle et al., 1994
CSGMT List	280	Evaluation of the rodent micronucleus assay in the screening of IARC carcinogens (Groups 1, 2A and 2B), including results from in vitro genotoxicity tests.	Morita et al., 1997
IARC List	935	Agents reviewed by the IARC monographs, volumes 1-100A, 2 April 2009. Group 1: Carcinogenic to humans (108), Group 2A: Probably carcinogenic to humans (63), Group 2B: Possibly carcinogenic to humans (248), Group 3: Not classifiable as to carcinogenicity to humans (515), Group 4: Probably not carcinogenic to humans (1).	IARC*
CPDB List	1547	Bioassay literature, with qualitative and quantitative analyses of both positive and negative experiments that have been published over the past 50 years in the general literature through 2001 and by the National Cancer Institute/National Toxicology Program through 2004.	CPDB**
NTP List	ca. 2300	NTP Results reports; Results, status and publication information on all NTP chemicals produced from NTP chemtrack system, 10 August 2000. http://www.predictive-toxicology.org/data/ntp/original_ntp_data.txt	NTP***
EU GHS List	ca. 4000	Regulation (EC) No 1272/2008 of the European parliament and of the council, List of harmonised classification and labelling of hazardous substances, 31 Decembar 2008. Consideration for carcinogenicity or mutagenicity categories 1A, 1B, and 2.	EU, 2008

*: http://monographs.iarc.fr/ENG/Classification/index.php
**: http://potency.berkeley.edu/
***: http://www.predictive-toxicology.org/data/ntp/original_ntp_data.txt

Table 2 Primary and Secondary Candidate Chemicals for International Validation Study of Alkaline Comet Assay

No.		Candidate chemical	CAS	IARC group	CPDB	EU GHS#	Comet§ (route/ species)	Other Genotoxicity Data§§ Ames	in vitro CA	in vivo MN##	Liver UDS	LD_{50}§§§ rat, po (mg/kg)	Chemical properties (class, mode of action, etc)	Price	Note
● **Genotoxic* carcinogens (Primary, 43; Secondary, 24; Final, 19)**															
1	✓	2-Acetylaminofluorene (2-AAF)	53-96-3	NL	+	NL	+ (ip, po/M)	+	+	+	+	mouse po 810	Aromatic amine, metabolic activation, bulky addact	JPY3800/1g (W)	Used in phase 4 step 1; Selection for inter-laboratory resproducibility
2		Acrylamide	79-06-1	2A	+	Carc. 1B Muta. 1B	+ (ip/M)	-	+	+	-	124	Amide, epoxide	JPY1200/25g (W)	Non-selection as final chemicals due to use in phase 2
3	✓	Acrylonitrile	107-13-1	2B	+	Carc. 1B	+ (ip/M)	+	+	+	-	78	Aliphatic cmpd, +ve in rat MN by iv treatment	JPY2100/500mL (W)	
4		Aflatoxin B1	1162-65-8	1	+	NL	+ (ip/M&R)	+	+	+	+	2.7	Polycyclic hydrocarbon, metabolic activation, various adducts	JPY45000/5mL (W)	Non-selection as secondary candidates due to expensive
5	✓	o-Anisidine (o-Anisidine HCl)	90-04-0 (134-29-2)	2B	+	Carc. 1B Muta. 2	+ (po/M&R)	+	+	-	-	1150	Aromatic amine, +ve in Ames with norharman, +ve in mouse TG (bladder, but -ve in liver)	JPY1350/25mL (W)	
6	✓	Azidothymidine	30516-87-1	2B	+	NL		-	+	+		3084	Heterocyclic cmpd, nucleoside analogue	JPY2500/100mg (W)	Expesive, but selected
7		Ganciclovir (alternative of azidothymidine)	82410-32-0	NL	NL	NL		-	+	+		mouse po >2000	Heterocyclic cmpd, nucleoside analogue, termination of DNA synthesis		Non-selection as final chemicals due to use of azidothymidine
8		Benomyl	17804-35-2	NL	NL	Muta. 1B		-	+	+		>10 g	Heterocyclic amine, aneugen,	JPY17400/250mg (S)	Non-selection as secondary candidates due to no evaluation of carcinogenicity by IARC or CPDB
9	✓	Benzene	71-43-2	1	+	Carc. 1A Muta. 1B	+ (po/M&R; ip/M)	-	+	+		930-1800	Aromatic cmpd	JPY780/500mL(W)	
10		Benzidine	92-87-5	1	+	Carc. 1A	+ (po/M&R)	+	+	+	+	309	Aromatic amine	JPY9200/5mg/mL (1 mL ample) (S)	Non-selection as secondary caldidates due to expensive and unhandy

No.		Candidate chemical	CAS	IARC group	CPDB	EU GHS[#]	Comet[$] (route/ species)	Other Genotoxicity Data[$$] Ames	In vitro CA	In vivo MN[##]	Liver UDS	LD_{50}[$$$] rat, po (mg/kg)	Chemical properties (class, mode of action, etc)	Price	Note
11		Benzo[a]pyrene	50-32-8	2A	+	Carc. 1B Muta. 1B	+ (ip, po/M)	+	+	+	-	rat sc 50	Polycyclic aromatic hydrocarbon, metabolic activation, epoxide, bulky adduct	JPY10000/100mg (W)	Non-selection as secondary candidates due to expensive
12	✓	Cadmium chloride	10108-64-2	1	+	Carc. 1B Muta. 1B	- (ip/M)	-	+	+		88	Inorganic metal cmpd, oxidative stress or DNA-repair inhibition?	JPY2500/25g (W)	
13		Chlorodibromomethane	124-48-1	3	+	NL	+ (po/M&R)	+	+	-	-	370	Aliphatic halide	JPY13500/10g (S)	Non-selection as secondary candidates due to simirarity to 1,2-dibromomethane and 1,3-dichloropropene
14	✓	*p*-Chloroaniline	106-47-8	2B	-	Carc. 1B	+ (po/M)	+	+	+		300	Aromatic amine, no adducts	JPY2400/25g (W)	
15	✓	Cisplatin	15663-27-1	2A	NL	NL	+ (ip/M)	+	+	+		25.8	Metal cmpd, cross-linking	JPY123400/5g (S)	Expensive, but selected.
16		Cyclophosphamide	50-18-0	1	+	NL	+ (ip, po/M)	+	+	+	-	100	Aziridine, metabolic activation, DNA alkylation	JPY27400/5g	Non-selection as secondary candidates due to no stock in the supplyier
17	✓	2,4-Diaminotoluene (2,4-DAT)	95-80-7	2B	+	Carc. 1B	+ (po/M&R)	+	+	Inc	+	590	Aromatic amine, metabolic activation, weak +ve in rat MN, but -ve in mouse MN	JPY2000/25g (W)	Used in phase 2; Selection for inter-laboratory reproducibility
18	✓	1,2-Dibromoethane	106-93-4	2A	+	Carc. 1B	+ (ip/M)	+	+	-	+	108	Aliphatic halide	JPY1600/25mL (W)	
19		3,3'-Dichlorobenzidine	91-94-1	2B	+	Carc. 1B	+ (po/M)	+		+	+	No data (>1000 mg/kg)	Aromatic amine	JPY12600/25g (W)	Non-selection as secondary candidates due to similarity to 2-AAF, o-anisidine, and others
20	✓	1,3-Dichloropropene	542-75-6	2B	+	Not classified	+ (ip/M)	+	-	-	-	470	Aliphatic halide	JPY11600/25g (S)	
21		Dichlorvos	62-73-7	2B	+	Not classified	+ (po/M)	+	+	-	-	17	Aliphatic halide	JPY4000/200mg (W)	Non-selection as secondary candidates due to expensive

No.		Candidate chemical	CAS	IARC group	CPDB	EU GHS[#]	Comet[$] (route/ species)	Other Genotoxicity Data[$$] Ames	in vitro CA	in vivo MN[##]	Liver UDS	LD_{50}[$$$] rat, po (mg/kg)	Chemical properties (class, mode of action, etc)	Price	Note
22		3,3'-Dimethoxybenzidine (3,3'-Dimethoxybenzidine 2HCl)	119-90-4 (20325-40-0)	2B	+	Carc. 1B	+ (po/M)	+	-	+	-	1920	Aromatic amine	JPY5000/25g (W)	Non-selection as secondary candidates due to similarity to 2-AAF, o-anisidine, and others
23	✓	1,2-Dimethylhydrazine HCl	306-37-6	2B	+	Carc. 1B	+ (ip, po/M; po/R)	+	+	+	+	100	Hydrazine	JPY8800/25g (T)	
24		7,12-Dimethylbenz[a]anthracene	57-97-6	NL	+	NL	+ (ip, po/M)	+	+	+	-	327	Polycyclic aromatic hydrocarbons, metabolic activation, bulky adduct	JPY35000/1g (W)	Non-selection as secondary candidates due to expensive
25		2,4-Dinitrotoluene	121-14-2	2B	+	Carc. 1B Muta. 2		+	-	-	+	268	Cyclic nitro cmpd	JPY1600/25g (W)	Non-selection as secondary candidates due to similarity to 2,4- and 2,6-DAT
26		2,6-Dintrotoluene	606-20-2	2B	+	Carc. 1B Muta. 2		+	+	-	+	mouse 621, 714	Cyclic nitro cmpd	JPY5500/25g (W)	Non-selection as secondary candidates due to similarity to 2,4- and 2,6-DAT
27		Etoposide	33419-42-0	2A	NL	NL		E	+	+		1784	Heterocyclic cmpd, topoisomerase inhibitor, -ve in TG	JPY10200/25mg (W)	Non-selection as secondary candidates due to expensive
28	✓	Hydroquinone	123-31-9	3	+	Carc. 2 Muta. 2		-	+	+		302	Aromatic cmpd, aneugen	JPY1300/25g (W)	
29		IQ	76180-96-6	2A	+	NL	+ (ip/M)	+	+	-		No data	Heterocyclic amine, metabolic activation	JPY31000/100mg (W)	Non-selection as secondary candidates due to expensive
30		4,4'-Methylenedianiline (4,4'-Methylenedianiline 2HCl)	101-77-9 (13552-44-8)	2B	+	Carc. 1B Muta. 2	+ (po/M)	+	+	+	-	No data	Aromatic amine, highest dose of 350 mg/kg in UDS test (rat, po)	JPY2300/25g (T)	Non-selection as secondary candidates due to similarity to 2-AAF, o-anisidine, and others
31	✓	Methyl methanesulfonate (MMS)	66-27-3	2B	+	NL	+ (ip/M&R)	+	+	+	+	225	Sulfonate, DNA alkylation	JPY8500/25g (W)	
32		*N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG)	70-25-7	2A	+	Carc. 1B	+ (ip, po/M)	+	+	+	Inc	90	*N*-nitoroso cmpd, DNA alkylation	JPY8000/5g (S)	Non-selection as secondary candidates due to similarity to MMS and MNU
33		*N*-Methyl-*N*-nitrosourea (MNU)	684-93-5	2A	+	NL		+	+	+		110	*N*-nitroso cmpd, DNA alkylation	JPY35800/25g (S)	Non-selection as final chemicals due to use in phase 3 and phase 4 step 1

No.		Candidate chemical	CAS	IARC group	CPDB	EU GHS[#]	Comet[$] (route/ species)	Other Genotoxicity Data[$$] Ames	In vitro CA	In vivo MN[##]	Liver UDS	LD_{50}[$$$] rat, po (mg/kg)	Chemical properties (class, mode of action, etc)	Price	Note
34		Mitomycin C (MMC)	50-07-7	2B	+	NL	+ (ip, po/M)	+	+	+		30	Aziridine, DNA alkylation, DNA cross linker	JPY6800/10mg (W)	Non-selection as final chemicals due to expensive and to use of busulfan as alternative
35		Daunomycin HCl (alternative of MMC)	23541-50-6	NL	NL	NL		+	+			290	Heterocyclic cmpd, DNA intercalation, inhibition of topoisomerase II, generation of oxygen free radicals		Non-selection as final chemicals due to use of busulfan as alternative
36	✓	Busulfan (Myleran) (alternative of MMC)	55-98-1	1	-	NL	+ (ip/M)	+	+	+		mouse 110	Sulfonate, bifunctional alkylating agent (DNA cross-linking)		
37		2-Nitropropane (2-NP)	79-46-9	2B	-	Carc. 1B	+ (ip/M)	+	-	-	+	720	Aliphatic cmpd	JPY1900/25g (T)	Non-selection as secondary candidates due to similarity to acrylonitrile
38		*N*-Nitrosodiethylamine (DEN)	55-18-5	2A	+	NL	+ (ip, po/M; ip/R)	+	+	-	+	220	*N*-nitroso cmpd, metabolic activation, DNA alkylation	JPY11300/1g, JPY21400/10mL (S)	Non-selection as secondary candidates due to similarity to DMN
39	✓	*N*-Nitrosodimethylamine (DMN)	62-75-9	2A	+	Carc. 1B	+ (ip/M)	+	+	+	+	26	*N*-nitroso cmpd, metabolic activation, DNA alkylation	JPY3400/1g (W)	
40	✓	4,4'-Oxydianiline	101-80-4	2B	+	Carc. 1B Muta. 1B	+ (po/M)	+	+	+	-	725	Aromatic amine	JPY2600/25g (W)	
41	✓	Sodium arsenite	7784-46-5	1	-	NL		-	+	+		41	Inorganic metal cmpd, oxidative stress or DNA-repair inhibition?	JPY1900/25g (W)	
42		Styrene-7,8-oxide	96-09-3	2A	+	Carc. 1B	+ (ip/M)	+	+	-		2000	Epoxide, DNA alkylation	JPY1800/25mL (W)	Non-selection as secondary candidates due to similarity to other DNA alkylators
43	✓	Thioacetamide	62-55-5	2B	+	Carc. 1B	+ (ip/M)	-	-	+		301	Amide, liver toxicity	JPY2500/25g (W)	Selection as also liver toxicant
● **Genotoxic non-carcinogens (Primary, 13; Secondary, 6; Final, 6)**															
44		4-Acetylaminofluorene	28322-02-3	NL	-	NL		+	-		-	mouse ip 364	Aromatic amine, analogue of 2-AAF		Non-selection as secondary candidates due to no suitable supplier

No.		Candidate chemical	CAS	IARC group	CPDB	EU GHS[#]	Comet[§] (route/ species)	Other Genotoxicity Data[§§] Ames	in vitro CA	in vivo MN[##]	Liver UDS	LD_{50}[###] rat, po (mg/kg)	Chemical properties (class, mode of action, etc)	Price	Note
45	✓	9-Aminoacridine (9-Aminoacridine Hydrochloride monohydrate)	90-45-9 (52417-22-8)	NL	NL	NL		+	+			mouse ip 68	Aromatic amine, DNA intercalation	JPY15000/250g (W)	
46	✓	*p*-Anisidine (*p*-Anisidine HCl)	104-94-9 (20265-97-8)	3	-	Not classified		+	+			1320	Aromatic amine, analogue of *o*-anisidine	JPY4600/100g (W)	
47		Methyl chloride	74-87-3	3	NL	Carc. 2		+			+	1800	Aliphatic halide, weak +ve in UDS by inhalation, water soluble gas	JPY3500/0.2 mg/mL (1 mL amplue) (S)	Non-selection as secondary candidates due to expensive and unhandly
48	✓	2,6-Diaminotoluene (2,6-DAT) (2,6-Diaminotoluene HCl)	823-40-5 (15481-70-6)	NL	-	Muta. 2	- (po/M&R)	+	+	+	Inc	>ca. 300	Aromatic amine, metaqbolic activation, analogue of 2,4-DAT	JPY6500/25g (W)	Used in phase 2; Selection for inter-laboratory reproducibility
49	✓	5-Fluorouracil (5-FU)	51-21-8	3	+	NL	- (ip/M)	-	+	+		230	Heterocyclic cmpd, nucleoside analogue, thymidylate synthase inhibitor, +ve only in mice in CPDB	JPY2100/1g (W)	
50	✓	8-Hydroxyquinoline	148-24-3	3	-	NL	- (po/M)	+	+	-	-	1200	Polycyclic hydrocarbon	JPY2450/25g (W)	
51		4-Nitro-o-phenylenediamine	99-56-9	3	-	NL	- (po/M)	+	+	-		681	Aromatic amine	JPY3000/25g (W)	Non-selection as secondary candidates due to similarity to 2,6-DAT
52		3-Nitropropionic acid	504-88-1	NL	-	NL		+	+			ip 67, mouse po 68	Aliphatic cmpd	JPY56600/10g (S)	Non-selection as secondary candidates due to no in vivo genotoixcity data
53		6-Mercaptopurine	50-44-2	3	-	NL		+	+	+		277	Polycyclic hydrocarbon, nucleoside analogue	JPY26600/100g (W)	Non-selection as secondary candidates due to similarity to 5-FU
54		Phenol	108-95-2	3	-	Muta. 2	+ (po/M)	-	+	+		317, 512	Aromatic cmpd, +ve in MN might be due to decreasing body temperature	JPY9400/25g (W)	Non-selection as secondary candidates due to questionable in vivo genotoxicity (herperseramia?)
55	✓	*p*-Phenylenediamine 2HCl p-Phenylenediamine	624-18-0 (106-50-3)	3	-	Not classified		+	+	-		147	Aromatic amine	JPY5500/25g (W)	
56		Thiabendazole	148-79-8	NL	-	Not classified	+ (po/M)	+	+	+		2080	Polycyclic hydrocarbon	JPY8200/200mg (W)	Non-selection as secondary candidates due to expensive

No.		Candidate chemical	CAS	IARC group	CPDB	EU GHS[#]	Comet[$] (route/ species)	Other Genotoxicity Data[$$] Ames	In vitro CA	In vivo MN[##]	Liver UDS	LD_{50}[$$$] rat, po (mg/kg)	Chemical properties (class, mode of action, etc)	Price	Note
● **Non-genotoxic carcinogens (Primary, 19; Secondary, 7; Final, 7)**															
57		Amitrole	61-82-5	3	+	Not classified		-	-	-		1100	Aromatic amine, hormonal effects and prulactin secretion	JPY9000/200mg (W)	Non-selection as secondary candidates due to expensive
58		Benzyl acetate	140-11-4	3	+	NL	+ (po/M&R)	-	-	-	-	2490	Aromatic cmpd	JPY900/25g (W)	Non-selection as secondary candidates due to similarity to di(2-ethylhexyl)phthalate and o-phenylphenol Na
59		Chloramphenicol	56-75-7	2A	-	NL	- (po/M)	-	+	Inc		2500	Cyclic nitro cmpd, DNA binding	JPY6000/200mg (W)	Non-selection as secondary candidates due to expensive
60	✓	Chloroform	67-66-3	2B	+	Carc. 2	- (po/M)	-	-	-	-	695	Allphatic halide, liver toxicity	JPY1700/100mL (W)	Selection as also liver toxicant
61	✓	Diethanolamine	111-42-2	2B	NL	NL		-	-	-		ca. 620	Allphatic cmpd, tumors of mouse liver and renal tubles due to choline deficiency (Kirkland et al., 2008)	JPY1350/25mL (W)	
62	✓	Di(2-ethylhexyl)phthalate	117-81-7	2B	+	Not classified	- (po/M)	-	-	-	-	30	Aromatic cmpd, peroxisome proliferation	JPY2600/1g (W)	
63		Diethylstilbestrol	56-53-1	1	+	NL	- (ip/M)	-	+	Inc		>3000	Aromatic cmpd	JPY8000/200mg (W)	Non-selection as secondary candidates due to expensive
64	✓	Ethanol	64-17-5	1	+	Not classified		-	-	-		7000	Allphatic cmpd, liver toxicity	JPY1260/500mL(W)	
65		Griseofluvin	126-07-8	2B	+	NL		-	+	-		>10g	Aromatic halide	JPY2700/5g (W)	Non-selection as secondary candidates due to no stocks in the supplier
66		Lead acetate	301-04-2	2B	+	Not classified	- (ip/M)	-	+	Inc		ip 150	Inorganic metal cmpd, vary weak +ve in mice and +ve in rat peripheral blood MN for 10 weeks treatment	JPY5700/25g (W)	Non-selection as secondary candidates due to questionable in vivo genotoxicity
67		Melamine	108-78-1	3	+	NL		-	-	-		3161	Aromatic amine, bladder and ureteral carcinomas due to calculus formation	JPY6000/100mg (W)	Non-selection as secondary candidates due to expensive

No.		Candidate chemical	CAS	IARC group	CPDB	EU GHS[#]	Comet[$] (route/ species)	Other Genotoxicity Data[$$] Ames	in vitro CA	in vivo MN[**]	Liver UDS	LD_{50}[$$$] rat, po (mg/kg)	Chemical properties (class, mode of action, etc)	Price	Note
68		Methapyrilene HCl	135-23-9	NL	+	NL		E	+		-	200	Aromatic amine		Non-selection as secondary candidates due to no suitable supplier
69	✓	Methyl carbamate	598-55-0	3	+	NL		-	-	-		2500	Amide, inflammation and hyperplasia resulting from bioaccumulatoin, toxic to liver	JPY3700/25g (W)	Selection as also liver toxicant
70	✓	o-Phenylphenol Na	132-27-4	2B	+	Not classified	+ (po/M&R)	-	+	-	-	591	Aromatic cmpd, -ve in vivo bone marrow CA test	JPY4400/100g (W)	
71		Polybrominated biphenyl	67774-32-7	2B	+	NL		-	-	-	-	>1000	Aromatic cmpd	JPY28500/1mL (W)	Non-selection as secondary candidates due to expensive
72		Progesterone	57-83-0	2B	NL	NL		-	-			>100, ip 327	Steroid, carcinogenicity hormonal effects, as progestins in IARC	JPY12500/5g (W)	Non-selection as secondary candidates due to no stocks in the supplyier
73	✓	Saccharin Na	128-44-9	3	+	NL	+ (?/M)	-	-	-		mouse po 17g	Heterocyclic cmpd, -ve in TG test and adduct, carcinogenicity to male rats only (bladder)	JPY1750/25g (W)	
74		Terephthalic acid	100-21-0	NL	NL	NL		-	+	-	-	>6400	Aromatic cmpd, rat bladder tumors will be secondary effect due to calculi	JPY1300/25g (W)	Non-selection as secondary candidates due to no evaluation of carcinogenicity by IARC or CPDB
75		Trichloroethylene	79-01-6	2A	+	Carc. 1B Muta. 2	- (po/M)	-	-	Inc	-	4920	Aliphatic halide	JPY1060/500mL (W)	Non-selection as secondary candidates due to slimilarity to chloroform
● **Non-genotoxic non-carcinogens (Primary, 15; Secondary, 9; Final, 6)**															
76		Allyl alcohol	107-18-6	NL	-	Not classified		-		-		64	Aliphatic cmpd, liver tox	JPY2800/25mL (W)	Non-selection as secondary candidates due to slimilarity to ethanol
77	✓	Ampicillin trihydrate	7177-48-2	3	-	NL		-	-	-		10g	Heterocyclic cmpd, beta-lactam antibiotics	JPY19100/25g (W)	
78	✓	o-Anthranilic acid	118-92-3	3	-	NL		-	+	-		5410	Aromatic cmpd	JPY2200/25g (W)	
79	✓	t-Butylhydroquinone	1948-33-0	NL	-	NL		-	+	-		700	Aromatic cmpd	JPY2000/25g (W)	

No.		Candidate chemical	CAS	IARC group	CPDB	EU GHS#	Comet§ (route/ species)	Other Genotoxicity Data§§ Ames	in vitro CA	in vivo MN##	Liver UDS	LD_{50}§§§ rat, po (mg/kg)	Chemical properties (class, mode of action, etc)	Price	Note
80		Camptothecine	7689-03-4	NL	NL	NL		-				153	Heterocyclic cmpd, topo I inhibitor	JPY4500/100mg (W)	Non-selection as secondary candidates due to expensive
81		Cycloheximide	66-81-9	NL	NL	Muta. 2		-				2; mouse 133	Heterocyclic cmpd, protein synthesis inhibitor	JPY140400/25g (W)	Non-selection as secondary candidates due to no evaluation of carcinogenicity by IARC or CPDB
82	✓	Ethionamide	536-33-4	3	+	NL		-	+			1320	Amide, carcinogenicity -ve in rats; possible thyroid tumours in mice (Kirkland et al., 2008), +ve only in female mice in CPDB, -ve in both species in NCI-CG-TR-46, liver toxicity	JPY12500/5g (W)	Selection as also liver toxicant
83		Eugenol	97-53-0	3	-	NL		-	+	-	-	1930	Aromatic cmpd	JPY8000/200mg (W)	Non-selection as secondary candidates due to expensive
84	✓	Isobutyraldehyde	78-84-2	NL	-	NL		-	+	-		>2000	Aliphatic cmpd	JPY1200/25mL (W)	
85		D-Mannitol	69-65-8	NL	-	NL		-	-	-		13500	Aliphatic cmpd	JPY1500/25g (W)	Non-selection as final chemicals due to use in phase 3 and phase 4 step 1
86	✓	D,L-Menthol	15356-70-4	NL	-	NL	- (po/M)	-	+	-		2900	Heterocyclic cmpd	JPY10400/10g (W)	
87		1-Nitropropane	108-03-2	NL	-	Not classified		-		-	-	455	Aliphatic cmpd, analogue of 2-NP	JPY2500/25mL (W)	Non-selection as secondary candidates due to non-selection of 2-NP
88	✓	Sodium chloride	7647-14-5	NL	-	NL		-	-	-		3000	Inorganic metal cmpd, stomach toxicity	JPY800/500g (W)	Selection as also stomach toxicant
89	✓	Trisodium EDTA (monohydrate)	150-38-9 (10378-22-0)	NL	-	NL	- (po/M)	-	-			2150	Aliphatic cmpd, chelating agent	JPY2400/50g (W)	
90		Triton X(-100)	9002-93-1	NL	NL	NL		-				1800	Aromatic cmpd, surfactant	JPY2200/500mL (W)	Non-selection as secondary candidates due to no evaluation of carcinogenicity by IARC or CPDB

No.	Candidate chemical	CAS	IARC group	CPDB	EU GHS#	Comet$ (route/ species)	Other Genotoxicity Data$$ Ames	in vitro CA	in vivo MN##	Liver UDS	$$$ LD_{50} rat, po (mg/kg)	Chemical properties (class, mode of action, etc)	Price	Note
● **Positive Control (Genotoxic carcinogen)**														
91	Ethyl methanesulfonate (EMS)	62-50-0	2B	NL	NL	+ (ip/M)	+	+	+		ip 350, mouse po 470	Sulfonate, DNA alkylation	JPY8000/25g (W)	

✓: Selected as final test chemicals

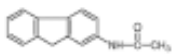
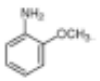
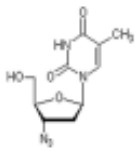
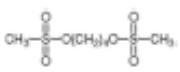
*: Genotoxic was defeined as Ames +ve and/or standard in vivo assay +ve.

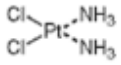
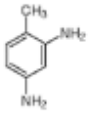
#: EU CLP Regulations (L353/1, 31.12.2008) for carcinogenicity and mutagenicity
##: Erythrocytes (bone marrow or peripheral blood) MN assay with mouse or rat

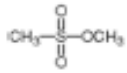
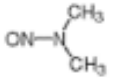
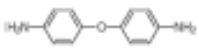
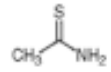
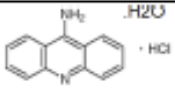
$: Data from Sasaki et al (2000), Sekihashi et al (2002), Kirkland et al (2006), and Kirkland and Speit (2008)
$$: Data from IARC monographs, Morita et al (1997), Sasaki et al (2000), Madle et al (1994), and Kirkland and Speit (2008)
$$$: Data from Registry of Toxic Effects of Chemical Substances (RTECS) and ChemIDplus

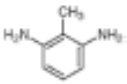
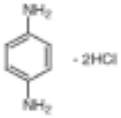
Abbreviations: CPDB, carcinogenic potency data base; CA, chromosomal aberration; MN, micronucleus; UDS, unscheduled DNA synthesis; TG, rodent transgenic mutation model
M, mice; R, rats; Ip, intraperitoneal injection; iv, intravenous injection; po, per os; NL, not listed;
E, equivocal; Inc, inconclusive (presence of both positive and negative findings); W, Wako; T, Tokyo Kasei; S, Sigma-Aldrich
GHS, globally harmonized system of classification and labelling of chemicals; Carc, carcinogenicity; Muta, mutagenicity;
+, positive; -, negative

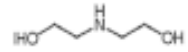
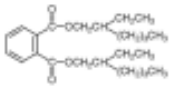
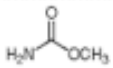
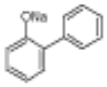
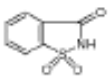
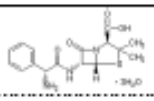
Table 3 Summary of in vivo Genotoxicity Data on Selected Final Test Chemicals for International Validation Study for Comet Assay

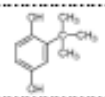
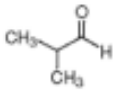
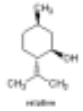
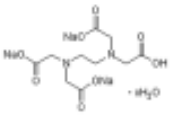
No.	Chemical [CAS]	Structure	Assay	Result	Animal	Route	Dose (mg/kg)	Note	Ref.
	Genotoxic* carcinogens (19)								
1	2-Acetylaminofluorene [53-96-3]		UDS	+	Rat	po	5, 50		1, 2
			MN	+	Rat	po	125-500 x 2d	Positive also in mouse	3
	<IARC, Not listed; CPDB, +ve>		Comet	+	Mouse	po	600	Positive in C, L, K, Lu	4, 5
			Liver TG	+	BigBlue mouse	Diet	72 x 28d		10, 65
			In vitro Ames/CA	+/+					12, 73
2	Acrylonitrile [107-13-1]		UDS	-	Rat	po	75, 60 x 5d		1, 6
			MN	-	Mouse	ip	6-45	Negative in both BM and PB	7
	<IARC, 2B; CPDB, +ve>		MN	-	Mouse	po	4-32	Negative in BM	7
			MN	-	Mouse	iv	10-40	Negative in both BM and PB	7
			MN	-	Rat	po	10-40	Negative in BM	7
			MN	+	Rat	iv	24.5-98	Positive in PB	7
			MN	+	Rat	iv	31-125 x 2d	Positive in BM, but negative in PB	3
			Comet	+	Rat	ip	30	Positive in S, Co, K, Bl, Lu	8
			Liver TG	Not done	MutaMouse	drink. water	for 28d	Negative in BM, Br, Lu, Sp lymp, testis	10
			In vitro Ames/CA	+/+					7, 73
3	o-Anisidine [90-04-0] (o-Anisidine HCl [134-29-2])		UDS	-	Rat	po	50-1104		1, 9
			MN	-	Mouse	ip	400-800		7
	<IARC, 2B; CPDB, +ve>		Comet	+	Rat	po	1000	Positive in K, Bl, Lu	8
			Liver TG	-	BigBlue Mouse	po	750 x 3d	Positive in Bl	10, 11
			In vitro Ames/CA	+/+					7, 73
4	Azidothymidine [30516-87-1]		MN	+	Mouse	po	500-2000 x 3d, 200-2000 x 3d		28
			MN	+	Mouse	ip	17 x 10 d/2 wks		29
	<IARC, 2B; CPDB, +ve>		MN	+	Rat	po	500 x 7d	Negative with single iv dose	62
			In vitro Ames/CA	-/+					12
5	Benzene [71-43-2]		MN	+	Rat	po	500-2000	Positive also in mouse	3
			Comet	+	Rat	po	2000	Positive in S, C, L, K, Ub, Lu, Br	4, 8
	<IARC, 1; CPDB, +ve>		Liver TG	-	BigBlue mouse	Inh	1350 ppm x 84d		10
			In vitro Ames/CA	-/+					7, 73
6	Busulfan (Myleran) [55-98-1]		MN	+	Mouse	ip	10-40		7
			Comet	+	Mouse	ip	40	Positive in C	4, 17
	<IARC, 1; CPDB, +ve>		In vitro Ames/CA	+/+					7, 73
7	Cadmium chloride [10108-64-2]		MN	+	Rat	po	15, 15 x 60d	as Cd dose?	30

No.	Chemical [CAS]	Structure	Assay	Result	Animal	Route	Dose (mg/kg)	Note	Ref.
			MN	+	Mouse	ip	1.9-7.6		31
	<IARC, 1; CPDB, +ve>		MN	-	Mouse	Drink. water	300 ppm for 7 days		**24**, 25
			Comet	-	Mouse	ip	1		**4**
			In vitro Ames/CA	-/+					**7, 73**
8	*p*-Chloroaniline [106-47-8]		MN	+	Mouse	po	300 x 3d	Negative in a test at 180 mg/kg (ref. 15)	**12, 13**, 14, **15**
			Comet	+	Mouse	po	200	Positive in S, C, L, Ub, Lu, Br	**4**, 16
	<IARC, 2B; CPDB, +ve>		In vitro Ames/CA	+/+					7, **12, 73**
9	Cisplatin [15663-27-1]		MN	+	Mouse	ip	0.03-10		7
			Comet	+	Mouse	ip	10	Positive in C, Lu, Br	**4**, 17
	<IARC, 2A; CPDB, Not listed>		Liver TG	+	LacZ mouse	ip	6		**10**, 66
			In vitro Ames/CA	+/+					7, **12, 73**
10	2,4-Diaminotoluene [95-80-7]		UDS	+	Rat	po	150 (+ve, Ref. 2); 300 (E or weak, Ref. 18)		**1**, 2, 18
			MN	-	Mouse	ip	30-240		7
	<IARC, 2B; CPDB, +ve>		MN	+	Rat (PVG)	po	150-300	Negative in F344 rats at 50-150 mg/kg	7, 18
			Comet	+	Rat	po	130	Positive in S, C, K, Br	8
			Liver Comet	-	Rat	po	25-100 x 29d	MN negative in BM	63
			Liver TG	+	BigBlue Mouse	po	66 x 12d		**10**, 19
			In vitro Ames/CA	+/+					7, **12, 73**
11	1,2-Dibromoethane [106-93-4]		UDS	+w	Rat	po	10-100		**1**, 20
			UDS	+	Rat	ip	100		**1**, 20
	<IARC, 2A; CPDB, +ve>		MN	-	Mouse	ip	25-150; 80-100 x 3d		7
			Comet	+	Mouse	ip	100	Positive in S, C, L, K, Ub, Lu	**4**, 21
			Liver TG	-	MutaMouse	ip	60, 16 x 5d		**10**, 67
			In vitro Ames/CA	+/+					**7, 73**
12	1,3-Dichloropropene [542-75-6]		UDS	-	Rat	po	125		**1**, 22
			MN	-	Mouse	ip	18.9-150		7
	<IARC, 2B; CPDB, +ve>		MN	-	Rat	po	125	Negative in BM, L, Spleen	76
			MN	+	Mouse (female)	po	187, 234	Negative in males at 140 and 180 mg/kg	77
			Comet	+	Mouse	ip	150	Positive in S, C, L, K, Ub, Lu, Br, BM	**4**, 21
			In vitro Ames/CA	+/-					**7, 73**
13	1,2-Dimethylhydrazine 2HCl [306-37-6] (1,2-Dimethylhydrazine [540-73-8])	 . 2HCl	UDS	+	Rat	po	20	as free base	**1**, 2
			MN	+	Rat	po	200 x 2d; 25-100 x 2d		3, 23
			MN	+	Mouse	ip	2.5-10 x 2d; 2.5-10 x 4d		7

No.	Chemical [CAS]	Structure	Assay	Result	Animal	Route	Dose (mg/kg)	Note	Ref.
	<IARC, 2A; CPDB, +ve>		Comet	+	Rat	po	100	Positive in S, C, L, K, Ub, Lu, Br, BM	4, 8
			Liver Comet	+	Rat	po	12.5-50, 1.25-5 x 29d	MN negative in BM & PB by 29d	63
			In vitro Ames/CA	+/+					7
14	Hydroquinone [123-31-9]		MN	+	Mouse	ip	30-100		12, 32
	<IARC, 3; CPDB, +ve>		MN	+	Mouse	po	60	Weaker than ip	33, 34
			In vitro Ames/CA	-/+					12, 73
15	Methyl methanesulfonate [66-27-3]		UDS	+	Rat	po	20-100		1, 2
			MN	+	Rat	po	36-144 x 2d		3
	<IARC, 2A; CPDB, +ve>		Comet	+	Rat	ip	60	Positive in S, C, L, K, Ub, Lu, Br, BM	4, 8
			Liver TG	+	Mouse	ip	100		10
			In vitro Ames/CA	+/+					7, 12
16	*N*-Nitrosodimethylamine [62-75-9]		UDS	+	Rat	po	10		1, 2
			MN	+	Mouse	po	25		24, 25
	<IARC,2A; CPDB, +ve>		Comet	+	Mouse	ip	6.25-50	Positive in S, C, L, K, Ub, Lu, Br, BM	4, 17
			Liver Comet	+	Rat	po	0.5-4 x 15d	MN negative in PB	63
			Liver TG	+	Mouse, Rat	po	Various doses and duration		10
			In vitro Ames/CA	+/+					7, 12, 73
17	4,4'-Oxydianiline [101-80-4]		UDS	-	Rat	po	40-725		1, 26
			MN	+	Mouse	ip	37.5-150 x 3d		27
	<IARC, 2B; CPDB, +ve>		Comet	+	Mouse	po	500	Positive in S, L, K, Ub, Lu, Br	4, 16
			In vitro Ames/CA	+/+					73
18	Sodium arsenite [7784-46-5]		MN	+	Mouse	ip	5-10	Positive in water, but negative in corn oil as vehichle	12, 35
	<IARC, 1; CPDB, -ve>		In vitro Ames/CA	-/+					7, 12
19	Thioacetamide [62-55-5]		MN	+	Mouse	po	50 -200		24, 57
			MN	+	Mouse	po	375-1500		68
	<IARC, 2B; CPDB, +ve>		Comet	+	Mouse	ip	200	Positive in S, C, Ub	4
			In vitro Ames/CA	-/-					7
	Genotoxic non-carcinogens (6)								
20	9-Aminoacridine hydrochloride monohydrate [52417-22-6]		No in vivo data						

No.	Chemical [CAS]	Structure	Assay	Result	Animal	Route	Dose (mg/kg)	Note	Ref.
	(9-Aminoacridine [90-45-9], 9-Aminoacridine HCl [134-50-9])		In vitro Ames/CA	+/+					73
	<IARC, Not listed; CPDB, Not listed>								
21	*p*-Anisidine [104-94-9] (*p*-Anisidine HCl [20265-97-6])		No in vivo data						
	<IARC, 3; CPDB, -ve>		In vitro Ames/CA	+/+					73
22	2,6-Diaminotoluene [823-40-5] (2,6-Diaminotoluene 2HCl [15481-70-6])		UDS	-	Rat	po	150; 150, 300		1, 2, 18
			UDS	+	Rat	po	1000, 1000 x 2d		1, 36
			MN	+	Mouse	ip	15.6-62.5 x 3d	as 2HCl salt	27
	<IARC, Not listed; CPDB, -ve>		MN	+w	Rat	po	300, 600	=< x2 from negative control	18
			MN	-	gpt Delta Rat	Diet	500 ppm (13 wks)		37
			Comet	-	Rat	po	250		4, 8
			Comet	+w	Rat	po	125-500 x 3d, 15-60 29d	MN negative in BM by 3 & 29d, positive in PB by 29d	63
			Liver TG	-	BigBlue Mouse	Diet	120 x 30&90d		10, 64
			In vitro Ames/CA	+/+					73
23	5-Fluorouracil [51-21-8]		MN	+	Rat (male)	ip	20-80		3
			MN	+	Rat	po	20, 40	4 wks old, Positive in PB, but negative in liver	38
	<IARC, 3; CPDB, +ve>		Comet	-	Mouse	ip	100		4, 39
			In vitro Ames/CA	-/+					73/74
24	8-Hydroxyquinoline [148-24-3]		UDS	-	Rat	po	100-500; 600, 600 x 2d		1, 36, 40
			MN	-	Mouse	ip	10.8-43 x 3d		27
	<IARC, 3; CPDB,-ve>		Comet	-	Mouse	po	600		4
			In vitro Ames/CA	+/+					73
25	*p*-Phenylenediamine 2HCl [624-18-0] (*p*-Phenylenediamine [106-50-3])		MN	-	Mouse	ip	20-100		41
			MN	-	Rat	po	300 x2d		78
			Comet	-	Rat	po	75		4, 8
	<IARC, 3; CPDB, -ve>		In vitro Ames/CA	+/+					73
	Non-genotoxic carcinogens (7)								
26	Chloroform [67-66-3]		UDS	-	Rat	po	40, 400		1, 2
			MN	-	Mouse	ip	238-952 x 2d		42, 43

No.	Chemical [CAS]	Structure	Assay	Result	Animal	Route	Dose (mg/kg)	Note	Ref.
			MN	+	Rat	po	480	In kidney cells, addition of folic acid (iv route) for increasing proliferation	42, 44
	<IARC, 2B; CPDB, +ve>		Comet	-	Mouse	po	400		4
			Liver TG	-	BigBlue mouse	Inh	154 x 10-180d		10
			In vitro Ames/CA	-/-					7, 73
27	Diethanolamine [111-42-2]		MN	-	Mouse	skin	80-1250 x 90d	MNNCE in blood	12, 58, 69
	<IARC, 2B; CPDB, Not listed>		In vitro Ames/CA	-/-					73
28	Di(2-ethylhexyl)phthalate [117-81-7]		UDS	-	Rat	po	500		1, 6
			MN	-	Mouse	ip	500-2000 x 2d		7
	<IARC, 2B; CPDB, +ve>		Comet	-	Mouse	po	2000		4
			TG	-	BigBlue mouse	Diet	360-720 x 120d		10, 45
			In vitro Ames/CA	-/-					12, 73
29	Ethanol [64-17-5]		MN	-	Mouse	Drink. Water	10 and 20% for 3 or 7 wks		46, 71
	<IARC, 1; CPDB, +ve>		In vitro Ames/CA	-/-					75
30	Methyl carbamate [598-55-0]		MN	-	Mouse	ip	500-2000, 2000-3000		12, 47
	<IARC, 3; CPDB, +ve>		In vitro Ames/CA	-/-					73
	o-Phenylphenol sodium salt [132-27-4] (o-Phenylphenol [90-43-7])		CA	-	Rat	Diet	0-2.0%		48, 49, 50
			CA	-	Mouse	po	250-4000; 50-800 x 5d		48
			MN	-	Rat	Diet	0-12500 ppm		52
	<IARC, 2B; CPDB, +ve; for sodium salt>		MN	+	Rat	Diet	80-12500 ppm	In urinaly bladder, as free base	48, 53
	<IARC, 3; CPDB, +ve; for free base>		Comet	+	Rat	po	2000	Positive in S, C, L, K, Ub, Lu, Br	4, 8
			Comet	-	Rat	po	2000		49, 54
			Comet	+	Mouse	po	100-2000	Positive in C, S, Ub, Lu, L, K	55
			In vitro Ames/CA	-/+					42
32	Saccharin [81-07-2]		CA	-	Mouse	?	4000	as Na salt	12, 56, 70
	(Saccharin sodium [128-44-9])		Comet	+	Mouse	po	100-2000	Positive in S, C as Na salt	49, 55
			Liver TG	-	BigBlue rat	Diet	Dose not specified (X 10d)	as Na salt	10
	<IARC, 3; CPDB, +ve for sodium salt, -ve for free base>		In vitro Ames/CA	-/-					12
	Non-genotoxic, non-carcinogens (8)								
33	Ampicillin trihydrate [7177-48-2] (Ampicillin [69-53-4])		MN	-	Rat	po	3000, 5000		12, 59

No.	Chemical [CAS]	Structure	Assay	Result	Animal	Route	Dose (mg/kg)	Note	Ref.
	<IARC, 3; CPDB, -ve>		In vitro Ames/CA	-/-					12, 73
34	*o*-Anthranilic acid [118-92-3]		MN	-	Mouse	ip	75-300, 150-600		12, 60
	<IARC, 3; CPDB, -ve>		In vitro Ames/CA	-/+					12, 73
35	*t*-Butylhydroquinone [1948-33-0]		MN	-	Mouse	ip	9-400 x 3d		12, 58
	<IARC, Not listed; CPDB, -ve>		In vitro Ames/CA	-/+					12, 73
36	Ethionamide [536-33-4]		No in vivo data						
	<IARC, 3; CPDB, +ve; NCI, -ve>		In vitro Ames/CA	-/+					12, 73
37	Isobutyraldehyde [78-84-2]		MN	-	Mouse	ip	39-1250 x 3d, 156-625 x 3d		12, 58
	<IARC, Not listed; CPDB, -ve>		MN	-	Rat	ip	313-1250 x 3d		12, 58
			In vitro Ames/CA	-/+					12, 73
38	D,L-Menthol [15356-70-4]		MN	-	Mouse	ip	250-1000 x 3d		27
			Comet	-	Mouse	po	2000		4
	<IARC, Not listed; CPDB, -ve>		In vitro Ames/CA	-/+					12, 73
39	Sodium chloride [7647-14-5]	NaCl	UDS	-	Rat	po	1000	Negative in stomach	61
			MN	-	Mouse	ip	2000		56
	<IARC, Not listed; CPDB, -ve>		In vitro Ames/CA	-/-					73
40	Trisodium EDTA monohydrate [10378-22-0]		MN	-	Mouse	po	500-2000	as disodium salt	72
	(EDTA [60-00-4], Trisodium EDTA trihaydarate [150-38-9], Disodium EDTA dihydrate [6381-92-6])		MN	-	Mouse	ip	166	as disodium salt	72
			MN	+	Mouse	ip	5-20	as disodium salt	72
			Comet	-	Mouse	po	6月22日		4
	<IARC, Not listed; CPDB, -ve>		In vitro Ames/CA	-/-					12, 73

*: Genotoxic compounds are definede as chemicals which are positive in the Ames test or standered in vivo genotoxicity test.

References (Bold is review doc.)

1. **Madle et al, Mutat. Res., 312, 263-285, 1994.**
2. Mirsalis et al, Environ. Mutagen., 4, 553-562, 1982.
3. Wakata et al., Environ. Mol. Mutagen., 32, 84-100, 1998.
4. **Sasaki, Crit Rev Toxicol. 30, 629-799, 2000.**
5. Sekihashi et al, Mutat. Res., 493, 39-54, 2001.
6. Butterworth et al, Environ. Mol. Mutagen., 20, 148-155, 1992.
7. Morita et al, Mutat. Res., 389, 1-122, 1997.
8. Sekihashi et al, Mutat. Res., 517, 53-74, 2002.
9. Ashby et al., Mutat. Res., 250, 115-133, 1991.
10. **Lambert et al, Mutat. Res., 590, 1-280, 2005.**

18. George and Westmoreland, Carcinogenesis, 12, 2233-2237, 1991.
19. Suter et al, Environ. Mol. Mutagen., 28, 354-362, 1996.
20. Working et al, Carcinogenesis, 7, 467-472, 1986.
21. Sasaki et al, Mutat. Res., 419, 13-20, 1998.
22. Ghiha et al, Toxicol. Appl. Pharmacol., 120, 120-125, 1993.
23. Takasawa et al, Mutat. Res., 698, 24-29, 2010.
24. Mavournin et al, Mutat. Res., 239, 29-80, 1990.
25. Watanabe et al, Mutat. Res., 97, 43-48, 1982.
26. Mirsalis et al., Environ. Mol. Mutagen., 14, 155-164, 1989.
27. Shelby et al, Environ. Mol. Mutagen., 21, 160-179, 1993.
28. Phillips et al, Environ. Mol. Mutagen., 18, 168-183, 1991.
29. Dertinger et al, Mutat. Res., 368, 301-307, 1996.
30. Celik et al, Toxicol. Mech. Methods, 19, 135-140, 2009.
31. Fahmy and Aly, J. Appl. Toxicol., 20, 231-238, 2000.
32. Adler and Kliesch, Mutat. Res., 234, 115-123, 1990.
33. IPCS/WHO, EHC 157, 1994.
34. Ciranni et al, Mutat. Res., 209, 23-28, 1988.
35. Twinwell et al, Environ. Health Perspect., 95, 205-210, 1991.
36. Allavena et al, Teratogen. Carcinogen. Mutagen., 12, 31-41, 1992.
37. Toyoda-Hokaiwado et al, Toxicol. Science, 114, 71-78, 2010.
38. Suzuki et al, Mutagenesis, 24, 9-16, 2009.
39. Sasaki et al, Mutata. Res., 391, 215-230, 1997.
40. Ashby et al, Environ. Mol. Mutagen., 14, 221-228, 1989.
41. Soler-Niedziela et al, Mutat. Res., 259, 43-48, 1991.
42. IARC, v73, 1999.
43. Gocik et al, Mutat. Res., 90, 91-109, 1981.
44. Robbiano et al, Mutat. Res., 413, 1-6, 1998.
45. Gunz et al, Environ. Mol. Mutagen., 21, 209-211, 1993.
46. Phillips and Jenkinson, Mutagenesis, 16, 91-101, 2001.
47. Shelby and Tice, Mutat. Res., 260, 311, 1991.
48. Brusick, Environ. Mol. Mutagen., 45, 460-481, 2005.
49. Brendler-Schwaab et al, Mutagenesis, 20, 245-254, 2005.
50. Yoshida and Hiraga, Ann Rep Tokyo Metr Res Lab PH, 33, 489-491, 1982.
51. Shirasu et al, Mutat. Res., 54, 227, 1978 (abstract)
52. Balakrishnan and Eastmond, Fd Chem Toxicol., 44, 1340-1347, 2006.
53. Balakrishan et al, Mutagenesis, 17, 89-93, 2002.
54. Bomhard et al, Crit. Rev. Toxicol., 32, 551-625, 2002.
55. Sasaki et al, Mutat. Res., 519, 103-119, 2002.
56. Ashby, Fd. Chem. Toxicol., 23, 507-519, 1985.
57. Chieli et al, Mutat. Res, 192, 141-143, 1987.
58. NTP Database, http://www.ntp-server.niehs.nih.gov
59. Stemp et al, Mutagenesis, 4, 439-445, 1989.
60. McFee et al, Environ. Mol. Mutagen., 14, 207-220, 1989.
61. Ohsawa et al, Mutat. Res., 287, 307-319, 1993.
62. IARC, V76, 2000
63. Rothfuss et al., Mutat. Res., 702, 40-69, 2010.
64. Hayward et al., Carcinogenesis, 16, 2429-2433, 1995
65. Shephard et al., Mutat. Res., 302, 91-96, 1993.
66. Louro et al., Environ. Mol. Mutagen., 40, 283-291, 2002.
67. Hachiya and Motohashi, Ind. Health, 38, 213-220, 2000.
68. Minkova, Mutat. Res., 352, 23-30, 1996.
69. Witt et al, Environ. Mol. Mutagen., 36, 163-194, 2000.
70. Leonard and Leonard, J. Environ. Path. Toxicol. 2, 1047, 1979.
71. Tates et al., Mutat. Res., 79, 285-288, 1980.
72. EU Risk Assessment Report, V49, 2004.
73. NTP Results Report, 2000.
(http://www.predictive-toxicology.org/data/ntp/original_ntp_data.txt)
(Similar to Gold and Zeiger, Handbook of carcinogenic potency and genotoxicity databases, 1997)
74. Ishidate et al., Mutat Res., 195, 151-213, 1988
75. IARC, v96, 2010.
76. Ghia et al., Toxicol. Apple. Pharmacol., 120, 120-125, 1983.
77. Kevekordes et al., Toxicol. Lett. 89, 35-42, 1996.
78. IARC, V16, 1978.

Table 4 Chemical class of the final slelcted chemicals for the Comet validation study

Chemical class	Genotoxic carcinogens	Genotoxic non-carcinogens	Non-genotoxic carcinogens	Non-genotoxic non-carcinogens
Aromatic compounds including aromatic aminesamines	7	4	2	2
Aliphatic compounds including aliphatic halides	3	0	3	2
Heterocyclic compounds	1	1	1	2
Metal compounds	3	0	0	1
Hydrazines	1	0	0	0
Sulfonates	2	0	0	0
N-nitroso compounds	1	0	0	0
Amides	1	0	1	1
Polycyclic hydrocarbons	0	1	0	0
Total (n=40)	19	6	7	8

Table 5 General principals of chemical selection in validation study

	Review items	Objective	Explanations
1	Purpose of the validation study	Derivation of maximum number of chemicals to be used	Suitable maximum number of chemicals should be derived based on main purpose of the validation study in addition to the chemicals which are used for validation of intra- and inter-laboratory reproducibility.
2	Selection of databases used	Determination of coverage of object chemicals	Suitable chemical database(s) should be selected based on main purpose of the validation study or study items. In general, candidate chemicals will be selected among the database(s).
3	Toxicological field or type of the validation study	Collection of candidate chemicals	Sufficient number of candidate chemicals should be collected based on the test system (e.g., in vitro, in vivo, animal species, toxicological field) of the validation study. The candidates sholud be examined in the suitable toxicological field, and there should be the test findings.
4	Chemical class	Scientific refinement of candidates	Broad range of chemical classes should be included if there is no limitation of the type of validatiuon study. It increases the universality of results in the validation study.
5	Chemical structure	Scientific refinement of candidates	Chemicals with similar structure will be increased or decreased, if necessary. Iomers will be also selected for comparison.
6	Toxicological mechanism or mode of action	Scientific refinement of candidates	Variety types of toxicological mechanism or mode of action should be included, if necessary.
7	Physical state and properties	Scientific refinement of candidates	Physical state (gas, liquid, or solid; color) or physical properties (e.g., solubility, volatility) should be considered. Certain chemicals will affect the study result. Physical state is important for easy handle of chemicals.
8	Sufficiency of existence information	Scientific refinement of candidates	Chemicals with sufficient information on toxicity, metabolism, stability, or etc should be selected.
9	Toxic values (e.g., LD_{50})	Practical refinement of candidates	Toxic value will affect amount of the test chemical, resuting in budget or availability limitation.
10	Availability	Practical refinement of candidates	Possibility of procurement should be considered, e.g., availability in the market, suitable purity, sufficient amount in same lot.
11	Easiness of handling of chemicals	Practical refinement of candidates	Easiness of handling chemicals should be considered based on package presentation, volume, storage condition, or regulations.
12	Price	Practical refinement of candidates	Total prices of chemicals including transportation cost should be set in the budget.

Appendix 4

Heisei 23 Year

EVALUATION OF PATHOLOGICAL SPECIMENS OBTAINED FROM COMET ASSAY VALIDATION STUDIES

Study Title: Evaluation of pathological specimens obtained from Comet assay validation studies

Name of Facility: Biosafety Research Center, Foods, Drugs and Pesticides
In charge of Study: Ryuichi Kato

FINAL REPORT

EVALUATION OF PATHOLOGICAL SPECIMENS OBTAINED FROM COMET ASSAY VALIDATION STUDIES
[NON-GLP]

Experiment No. D807 (079-519)

February 20, 2012

SPONSOR
National Institute of Health Sciences

Biosafety Research Center, Foods, Drugs and Pesticides

Exp. No. D807 (079-519)
FINAL REPORT

SIGNATURE OF STUDY DIRECTOR AND STUDY PERSONNEL AND DATE

Study Title: Evaluation of pathological specimens obtained from Comet assay validation studies

Study No. D807 (079-519)

Study Director:

_____ Date: _____

Jin Tanaka
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_____ Date: _____

Atsushi Shiga
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Exp. No. D807 (079-519)
FINAL REPORT

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Exp. No. D807 (079-519)
FINAL REPORT

1. STUDY TITLE

Evaluation of pathological specimens obtained from Comet assay validation studies

2. PURPOSE

To evaluate the pathological specimens of liver and stomach tissues obtained from International *in vivo* Comet assay validation studies led by JaCVAM (Japanese Center for the Validation of Alternative Methods).

3. EXPERIMENT NUMBER

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8. PATHOLOGICAL SPECIMENS

Pathological specimens of 29 out of the 40 chemicals which were examined in the phase IV-2 validation studies of International Comet assay validation were provided favorably from the participating facilities.

9. Results of Comet assay

Test substance (Code No.)		Organ	Judgment*	Lab.
A4201		Liver	Positive	BioReliance
		Stomach	Negative	
A4202		Liver and stomach	Negative	BioReliance
A4203		Liver and stomach	Negative	BioReliance
A4204		Liver and stomach	Positive	Integrated Laboratory Systems (ILS)
A4206		Liver and stomach	Negative	ILS
A4210		Liver and stomach	Positive	Huntingdon Life Science (HLS)
A4211		Liver and stomach	Negative	HLS
A4212		Liver and stomach	Negative	HLS
A4213		Liver and stomach	Positive	Novartis
A4214		Liver	Positive	Novartis
		Stomach	Negative	
A4215		Liver and stomach	Negative	Novartis
A4219		Liver and stomach	Equivocal	ILS
A4222		Liver and stomach	Negative	Janssen Research & Development (J R&D)
A4223		Liver and stomach	Negative	J R&D
A4224	1st	Liver	Equivocal	J R&D
		Stomach	Negative	
	2nd	Liver	Positive	
		Stomach	Negative	
A4226		Liver and stomach	Negative	ILS
A4227		Liver and stomach	Negative	ILS
A4228		Liver and stomach	Negative	Food and Drug Safety Center (FDSC)
A4229		Liver and stomach	Negative	FDSC
A4230		Liver and stomach	Negative	FDSC
A4231		Liver and stomach	Positive	Mitsubishi Chemical Medience (MCM)
A4232		Liver and stomach	Negative	MCM
A4233		Liver and stomach	Negative	MCM
A4234		Liver and stomach	Negative	Sumitomo Chemical (SC)
A4235		Liver and stomach	Negative	SC
A4236		Liver and stomach	Negative	SC
A4237		Liver and stomach	Positive	The Institute of Environmental Toxicology (IET)
A4238		Liver and stomach	Negative	IET
A4239		Liver and stomach	Negative	IET

*: Judgment by the laboratory conducted the Comet assay(s)

10. PATHOLOGICAL EXAMINATION

For each test substance, the specimens (histological slides) of the negative control group and high-dose group were observed. For the following test substances (code; A4210 to A4212, A4229 and A4230), only the specimens of high-dose groups were observed due to the lack of the specimens of the negative control groups. When the high-dose specimens could not be prepared due to the toxicity of the test substance, etc., the specimens of the available highest dosages were observed.

In the A4237-treated stomach, a statistically significant increase in the % tail DNA was observed in only middle-dose group as compared with the negative control group. Therefore, the stomach specimens of A4237-middle-dose group were also observed.

Since the stomach-tissue slides of the A4237-high-dose group were not prepared, hematoxylin-eosin (HE) stained slides were made from the formalin-fixed stomach tissue of the high-dose group.

11. RESULTS

11.1. A4201

The results are shown in Table 1 and Appendix 1.

11.1.1. Liver

As histological findings considered to be the effect of the test substance, decrease of hepatocyte glycogen (5/5), vacuolation of perilobular hepatocytes (4/5), eosinophilic change of perilobular hepatocytes (1/5) and anisonucleosis of perilobular hepatocytes (5/5) were observed in the 200 mg/kg treatment group.

11.1.2. Stomach

Histological findings relating to the test substance treatment were not observed.

11.2. A4202

The results are shown in Table 2 and Appendix 2.

11.2.1. Liver

As histological findings considered to be the effect of the test substance, decrease of hepatocyte glycogen (3/5), vacuolar degeneration of centrilobular hepatocytes (5/5), hypertrophy of perilobular of hepatocytes (5/5), single cell necrosis of centrilobular hepatocytes (1/5), focal necrosis of hepatocytes (2/5), increase in mitosis of perilobular hepatocytes (4/5) and anisonucleosis of perilobular hepatocytes (5/5) were observed in the 500 mg/kg treatment group.

11.2.2. Stomach

As histological finding considered to be the effect of the test substance, single cell necrosis of chief cells (3/5) was observed in the 500 mg/kg treatment group.

11.3. A4203

The results are shown in Table 3 and Appendix 3.

11.3.1. Liver

As histological finding considered to be the effect of the test substance, decrease of hepatocyte glycogen (2/5) was observed in the 40 mg/kg treatment group.

11.3.2. Stomach

Histological findings relating to the test substance treatment were not observed.

11.4. A4204

The results are shown in Table 4 and Appendix 4.

11.4.1. Liver

Histological findings relating to the test substance treatment were not observed.

11.4.2. Stomach

Histological findings relating to the test substance treatment were not observed.

11.5. A4206

The results are shown in Table 5 and Appendix 5.

11.5.1. Liver

Histological findings relating to the test substance treatment were not observed.

Focal necrosis of hepatocyte (1/5) was observed in the test substance treatment group. However, it was judged to be spontaneous since the incidence of this lesion was similar to that observed in non-treatment animals.

11.5.2. Stomach

Histological findings relating to the test substance treatment were not observed.

11.6. A4210

The results are shown in Table 6 and Appendix 6.

11.6.1. Liver

As histological findings considered to be the effect of the test substance, decrease of hepatocyte glycogen (5/5), microvesicular vacuolation of hepatocytes (2/5) and single cell necrosis of centrilobular hepatocytes (5/5) were observed in the 150 mg/kg treatment group.

11.6.2. Stomach

Histological findings relating to the test substance treatment were not observed.

11.7. A4211

The results are shown in Table 7 and Appendix 7.

11.7.1. Liver

As histological findings considered to be the effect of the test substance, increase in mitosis of hepatocytes (4/5) and anisonucleosis of hepatocytes (3/5) were observed in the 525 mg/kg treatment group.

11.7.2. Stomach

Histological findings relating to the test substance treatment were not observed.

11.8. A4212

The results are shown in Table 8 and Appendix 8.

11.8.1. Liver

As histological findings considered to be the effect of the test substance, decrease of hepatocyte glycogen (5/5), diffuse hypertrophy of hepatocytes (5/5) and shrinkage of hepatocyte nuclei (5/5) were observed in the 2000 mg/kg treatment group.

11.8.2. Stomach

Histological findings relating to the test substance treatment were not observed.

11.9. A4213

The results are shown in Table 9 and Appendix 9.

11.9.1. Liver

As histological finding considered to be the effect of the test substance, pericholangiolar neutrophil infiltration (5/5) was observed in the 80 mg/kg treatment group.

11.9.2. Stomach

As histological findings considered to be the effect of the test substance, erosion (2/5) and proliferation of surface epithelial cells (2/5) were observed in the 80 mg/kg treatment group.

11.10. A4214

The results are shown in Table 10 and Appendix 10.

11.10.1. Liver

As histological finding considered to be the effect of the test substance, decrease of

hepatocyte glycogen (1/5) was observed in the 600 mg/kg treatment group.

11.10.2. Stomach

Histological findings relating to the test substance treatment were not observed.

11.11. A4215

The results are shown in Table 11 and Appendix 11.

11.11.1. Liver

Histological findings relating to the test substance treatment were not observed.

11.11.2. Stomach

Histological findings relating to the test substance treatment were not observed.

Single cell necrosis (1/5) was observed in the test substance treatment group. However, it was judged to be spontaneous since the incidence of this lesion was similar to that observed in non-treatment animals.

11.12. A4219

The results are shown in Table 12 and Appendix 12.

11.12.1. Liver

As histological findings considered to be the effect of the test substance, nuclear enlargement of hepatocytes (5/5), nuclear enlargement of bile duct epithelial cells (3/5), decrease of hepatocyte glycogen (5/5) and single cell necrosis of hepatocytes (1/5) were observed in the 30.0 mg/kg treatment group.

11.12.2. Stomach

Histological findings relating to the test substance treatment were not observed.

Single cell necrosis (1/5) was observed in the test substance treatment group. However, it was judged to be spontaneous since the incidence of this lesion was similar to that observed in non-treatment animals.

11.13. A4222

The results are shown in Table 13 and Appendix 13.

11.13.1. Liver

As histological findings considered to be the effect of the test substance, decrease of hepatocyte glycogen (2/3) and hypertrophy of perilobular of hepatocytes (3/3) were observed in the 100 mg/kg treatment group.

11.13.2. Stomach

Histological findings relating to the test substance treatment were not observed.

11.14. A4223

The results are shown in Table 14 and Appendix 14.

11.14.1. Liver

As histological findings considered to be the effect of the test substance, decrease of hepatocyte glycogen (1/5), increase in mitosis of hepatocytes (3/5) and anisonucleosis of hepatocytes (3/5) were observed in the 1000 mg/kg treatment group.

11.14.2. Stomach

Histological findings relating to the test substance treatment were not observed.

11.15. A4224

11.15.1. 1st

The results are shown in Table 15-1 and Appendix 15-1.

11.15.1.1. Liver

As histological findings considered to be the effect of the test substance, decrease of hepatocyte glycogen (5/5), eosinophilic change of hepatocytes (5/5), single cell necrosis of hepatocytes (5/5), hypertrophy of perilobular of hepatocytes (3/5), hypertrophy of sinusoidal cells (5/5), anisonucleosis of hepatocytes (5/5) and proliferation of bile duct (4/5) were observed in the 150 mg/kg treatment group.

Focal fibrosis (1/5) was observed in the test substance treatment group. However, it was judged to be spontaneous since the incidence of this lesion was similar to that observed in non-treatment animals.

11.15.1.2. Stomach

Histological findings relating to the test substance treatment were not observed.

11.15.2. 2nd

The results are shown in Table 15-2 and Appendix 15-2.

11.15.2.1. Liver

As histological findings considered to be the effect of the test substance, decrease of hepatocyte glycogen (5/5), eosinophilic change of hepatocytes (5/5), single cell necrosis of hepatocytes (5/5), hypertrophy of perilobular of hepatocytes (3/5), hypertrophy of sinusoidal cells (5/5), anisonucleosis of hepatocytes (5/5) and proliferation of bile duct (5/5) were observed in the 200 mg/kg treatment group.

11.15.2.2. Stomach

As histological finding considered to be the effect of the test substance, focal edema (1/5) was observed in the 200 mg/kg treatment group.

11.16. A4226

The results are shown in Table 16 and Appendix 16.

11.16.1. Liver

As histological findings considered to be the effect of the test substance, decrease of hepatocyte glycogen (1/5) and increase in mitosis of hepatocytes (2/5) were observed in the 600 mg/kg treatment group.

11.16.2. Stomach

Histological findings relating to the test substance treatment were not observed.

11.17. A4227

The results are shown in Table 17 and Appendix 17.

11.17.1. Liver

Histological findings relating to the test substance treatment were not observed.

11.17.2. Stomach

Focal inflammation (1/5) was observed in the test substance treatment group. However, it was judged to be spontaneous since the incidence of this lesion was similar to that observed in non-treatment animals.

11.18. A4228

The results are shown in Table 18 and Appendix 18.

11.18.1. Liver

Histological findings relating to the test substance treatment were not observed.

11.18.2. Stomach

Histological findings relating to the test substance treatment were not observed.

11.19. A4229

The results are shown in Table 19 and Appendix 19.

11.19.1. Liver

As histological finding considered to be the effect of the test substance, decrease of hepatocyte glycogen (4/5) was observed in the 62.5 mg/kg treatment group.

Hypertrophy of sinusoidal cells (1/5) was observed in the test substance treatment

group. However, it was judged to be spontaneous since the incidence of this lesion was similar to that observed in non-treatment animals.

11.19.2. Stomach

Histological findings relating to the test substance treatment were not observed.

11.20. A4230

The results are shown in Table 20 and Appendix 20.

11.20.1. Liver

Histological findings relating to the test substance treatment were not observed.

11.20.2. Stomach

Histological findings relating to the test substance treatment were not observed.

11.21. A4231

The results are shown in Table 21 and Appendix 21.

11.21.1. Liver

As histological findings considered to be the effect of the test substance, decrease of hepatocyte glycogen (4/5), vacuolation of perilobular hepatocytes (3/5), single cell necrosis of centrilobular hepatocytes (4/5), necrosis of centrilobular hepatocytes (1/5), anisonucleosis of hepatocytes (5/5) and increase in mitosis of hepatocytes (2/5) were observed in the 100 mg/kg treatment group.

Focal necrosis of hepatocyte (1/5) was observed in the test substance treatment group. However, it was judged to be spontaneous since the incidence of this lesion was similar to that observed in non-treatment animals.

11.21.2. Stomach

As histological finding considered to be the effect of the test substance, single cell necrosis of chief cells (1/5) was observed in the 100 mg/kg treatment group.

11.22. A4232

The results are shown in Table 22 and Appendix 22.

11.22.1. Liver

As histological findings considered to be the effect of the test substance, decrease of hepatocyte glycogen (4/5), erythrophagia of Kupper cells (2/5), hypertrophy of perilobular hepatocytes (5/5) and increase in mitosis of hepatocytes (3/5) were observed in the 500 mg/kg treatment group.

Focal necrosis of hepatocytes (1/5) was observed in the test substance treatment

group. However, it was judged to be spontaneous since the incidence of this lesion was similar to that observed in non-treatment animals.

11.22.2. Stomach

Histological findings relating to the test substance treatment were not observed.

11.23. A4233

The results are shown in Table 23 and Appendix 23.

11.23.1. Liver

As histological findings considered to be the effect of the test substance, decrease of hepatocyte glycogen (5/5) and hypertrophy of perilobular hepatocytes (5/5) were observed in the 2000 mg/kg treatment group.

11.23.2. Stomach

Histological findings relating to the test substance treatment were not observed.

11.24. A4234

The results are shown in Table 24 and Appendix 24.

11.24.1. Liver

Histological findings relating to the test substance treatment were not observed.

11.24.2. Stomach

Histological findings relating to the test substance treatment were not observed.

11.25. A4235

The results are shown in Table 25 and Appendix 25.

11.25.1. Liver

As histological findings considered to be the effect of the test substance, decrease of hepatocyte glycogen (5/5), diffuse hypertrophy of hepatocytes (2/5) and increase in mitosis of hepatocytes (5/5) were observed in the 2000 mg/kg treatment group.

11.25.2. Stomach

Histological findings relating to the test substance treatment were not observed.

11.26. A4236

The results are shown in Table 26 and Appendix 26.

11.26.1. Liver

As histological finding considered to be the effect of the test substance, decrease of hepatocyte glycogen (4/5) was observed in the 2000 mg/kg treatment group.

11.26.2. Stomach

Histological findings relating to the test substance treatment were not observed.

11.27. A4237

The results are shown in Table 27 and Appendix 27.

11.27.1. Liver

As histological finding considered to be the effect of the test substance, decrease of hepatocyte glycogen (5/5) was observed in the 80 mg/kg treatment group.

11.27.2. Stomach

Histological findings relating to the test substance treatment were not observed.

11.28. A4238

The results are shown in Table 28 and Appendix 28.

11.28.1. Liver

As histological findings considered to be the effect of the test substance, centrilobular hemorrhage (1/5), decrease of hepatocyte glycogen (3/5), granular degeneration of hepatocytes (5/5), single cell necrosis of centrilobular hepatocytes (1/5), necrosis of centrilobular hepatocytes (1/5), increase in mitosis of hepatocytes (1/5) and infiltration of inflammatory cells (5/5) were observed in the 500 mg/kg treatment group.

11.28.2. Stomach

Histological findings relating to the test substance treatment were not observed.

Focal necrosis (1/5) was observed in the test substance treatment group. However, it was judged to be spontaneous since the incidence of this lesion was similar to that observed in non-treatment animals.

11.29. A4239

The results are shown in Table 29 and Appendix 29.

11.29.1. Liver

As histological findings considered to be the effect of the test substance, decrease of hepatocyte glycogen (2/5), shrinkage of hepatocytes (5/5), anisonucleosis of hepatocytes (3/5) and increase in mitosis of hepatocytes (5/5) were observed in the 2000 mg/kg treatment group.

11.29.2. Stomach

Histological findings relating to the test substance treatment were not observed.

Erosion (1/5) and focal necrosis (1/5) were observed in the test substance treatment

group. However, it was judged to be spontaneous since the incidence of this lesion was similar to that observed in non-treatment animals.

12. DISCUSSION

The pathological specimens of liver and stomach tissues obtained from International *in vivo* Comet assay validation studies were evaluated.

The pathological specimens of liver and stomach tissues obtained from International *in vivo* Comet assay validation studies were examined. The table (Table 30), which contains the both results of Comet assay and histological findings of the test substance, was made to confirm the relationship between the both results. The Comet-positive test substances specifically induced eosinophilic change of perilobular hepatocytes, pericholangiolar neutrophil infiltration, vacuolation of perilobular hepatocytes and microvesicular vacuolation of hepatocytes. It is not considered that these findings affect the results of each Comet assay. The relationship between the necrosis of cells detected by pathological examination and the results of Comet assay (% tail DNA and hedgehog/cloud) was examined. However, clear relationship could not be confirmed.

13. ARCHIVES

The following items of this study will be retained in the archives of BSRC for 10 years after the final report is made. Any subsequent retention will be decided after the discussion between the Sponsor and BSRC.

- Final report (copy)
- Other data related to the study

Table 1 Summary of Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4201 Test facility : BioReliance

Organs	Histological findings	Group	1	4
		Dose (mg/kg)	0	200
		No. of animals	5	5
Liver				
	no remarkable change		5	0
	decrease, glycogen, hepatocyte		0	5
	vacuolation, hepatocyte, perilobular		0	4
	eosinophilic change, hepatocyte, perilobular		0	1
	anisonucleosis, hepatocyte		0	5
Glandular stomach				
	no remarkable change		5	5

Table 2 Summary of Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4202 Test facility : BioReliance

Organs	Histological findings	Group	1	4
		Dose (mg/kg)	0	500
		No. of animals	5	5
Liver				
	no remarkable change		4	0
	decrease, glycogen, hepatocyte		0	3
	degeneration, vacuolar, hepatocyte, centrilobular		0	5
	hypertrophy, hepatocyte, perilobular		0	5
	single cell necrosis, hepatocyte, centrilobular		0	1
	necrosis, hepatocyte, focal		0	2
	increase in mitosis, hepatocyte, panlobular		1	0
	increase in mitosis, hepatocyte, perilobular		0	4
	anisonucleosis, hepatocyte, perilobular		0	5
Glandular stomach				
	no remarkable change		5	3
	single cell necrosis, chief cell		0	2

Table 3 Summary of Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4203 Test facility : BioReliance

Organs	Histological findings	Group	1	4
		Dose (mg/kg)	0	40
		No. of animals	5	5
Liver				
	no remarkable change		5	3
	decrease, glycogen, hepatocyte		0	2
Glandular stomach				
	no remarkable change		5	5

Table 4 Summary of Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4204 Test facility : ILS Inc.

		Group	1	4
		Dose (mg/kg)	0	2.5
Organs	Histological findings	No. of animals	5	5
Liver				
	no remarkable change		4	5
	capsulitis, focal		1	0
Glandular stomach				
	no remarkable change		5	5

Table 5 Summary of Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4206 Test facility : ILS Inc.

		Group	1	4
		Dose (mg/kg)	0	6.25
Organs	Histological findings	No. of animals	5	5
Liver				
	no remarkable change		4	4
	single cell necrosis, hepatocyte		1	0
	necrosis, hepatocyte, focal		0	1
Glandular stomach				
	no remarkable change		5	5

Table 6 Sumary of Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4210 Test facility : Huntingdon Life Science

		Group	4
		Dose (mg/kg)	150
Organs	Histological findings	No. of animals	5
Liver			
	no remarkable change		0
	decrease, glycogen, hepatocyte		5
	vacuolation, microvesicular, hepatocyte		2
	single cell necrosis, hepatocyte, centrilobular		5
Glandular stomach			
	no remarkable change		5

Table 7 Sumary of Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4211 Test facility : Huntingdon Life Science

Organs	Histological findings	Group	4
		Dose (mg/kg)	525
		No. of animals	5
Liver			
	no remarkable change		1
	increase in mitosis, hepatocyte		4
	anisonucleosis, hepatocyte		3
Glandular stomach			
	no remarkable change		5

Table 8 Sumary of Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4212 Test facility : Huntingdon Life Science

Organs	Histological findings	Group	4
		Dose (mg/kg)	2000
		No. of animals	5
Liver			
	no remarkable change		0
	decrease, glycogen, hepatocyte		5
	hypertrophy, hepatocyte, diffuse		5
	shrinkage, nucleus, hepatocyte		5
Glandular stomach			
	no remarkable change		5

Table 9 Summary of Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4213 Test facility : Novartis Pharmaceuticals Corporation

		Group	1	4
		Dose (mg/kg)	0	80
Organs	Histological findings	No. of animals	5	5
Liver				
	no remarkable change		5	0
	infiltration, neutrophil, pericholangiolar		0	5
Glandular stomach				
	no remarkable change		5	2
	erosion		0	2
	proliferation, surface epithelial cell		0	2

Table 10 Summary of Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4214 Test facility : Novartis Pharmaceuticals Corporation

		Group	1	4
		Dose (mg/kg)	0	600
Organs	Histological findings	No. of animals	5	5
Liver				
	no remarkable change		5	4
	decrease, glycogen, hepatocyte		0	1
Glandular stomach				
	no remarkable change		5	5

Table 11 Summary of Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4215 Test facility : Novartis Pharmaceuticals Corporation

		Group	1	4
		Dose (mg/kg)	0	100
Organs	Histological findings	No. of animals	5	5
Liver				
	no remarkable change		5	5
Glandular stomach				
	no remarkable change		5	4
	single cell necrosis		0	1

Table 12 Summary of Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4219 Test facility : ILS Inc.

		Group	1	4
		Dose (mg/kg)	0	30.0
Organs	Histological findings	No. of animals	5	5
Liver				
	no remarkable change		5	0
	nuclear enlargement, hepatocyte		0	5
	nuclear enlargement, bile duct epithelium		0	3
	decrease, glycogen, hepatocyte		0	5
	single cell necrosis, hepatocyte		0	1
Glandular stomach				
	no remarkable change		5	4
	single cell necrosis		0	1

Table 13 Summary of Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4222 Test facility : Janssen Pharmaceuticals, Inc.

		Group	1	4
		Dose (mg/kg)	0	100
Organs	Histological findings	No. of animals	5	3
Liver				
	no remarkable change		5	0
	decrease, glycogen, hepatocyte		0	2
	hypertrophy, hepatocyte, perilobular		0	3
Glandular stomach				
	no remarkable change		5	3

Table 14 Summary of Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4223 Test facility : Janssen Pharmaceuticals, Inc.

		Group	1	4
		Dose (mg/kg)	0	1000
Organs	Histological findings	No. of animals	5	5
Liver				
	no remarkable change		4	1
	decrease, glycogen, hepatocyte		0	1
	necrosis, hepatocyte, focal		1	0
	increase in mitosis, hepatocyte		0	3
	anisonucleosis, hepatocyte		0	3
Glandular stomach				
	no remarkable change		5	5

Table 15-1 Summary of Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4224-first study Test facility : Janssen Pharmaceuticals, Inc.

		Group	1	4
		Dose (mg/kg)	0	150
Organs	Histological findings	No. of animals	5	5
Liver				
	no remarkable change		5	0
	decrease, glycogen, hepatocyte		0	5
	eosinophilic change, hepatocyte		0	5
	single cell necrosis, hepatocyte		0	5
	hypertrophy, hepatocyte, perilobular		0	3
	hypertrophy, sinusoidal cell		0	5
	anisonucleosis, hepatocyte		0	5
	proliferation, bile duct		0	4
	fibrosis, focal		0	1
Glandular stomach				
	no remarkable change		5	5

Table 15-2 Summary of Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4224-second study Test facility : Janssen Pharmaceuticals, Inc.

		Group	1	4
		Dose (mg/kg)	0	200
Organs	Histological findings	No. of animals	5	5
Liver				
	no remarkable change		5	0
	decrease, glycogen, hepatocyte		0	5
	eosinophilic change, hepatocyte		0	5
	single cell necrosis, hepatocyte		0	5
	hypertrophy, hepatocyte, perilobular		0	3
	hypertrophy, sinusoidal cell		0	5
	anisonucleosis, hepatocyte		0	5
	proliferation, bile duct		0	5
Glandular stomach				
	no remarkable change		5	4
	edema, focal		0	1

Table 16 Summary of Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4226 Test facility : ILS Inc.

Organs	Histological findings	Group	1	4
		Dose (mg/kg)	0	600
		No. of animals	5	5
Liver				
	no remarkable change		5	2
	decrease, glycogen, hepatocyte		0	1
	increase in mitosis, hepatocyte		0	2
Glandular stomach				
	no remarkable change		5	5

Table 17 Summary of Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4227 Test facility : ILS Inc.

Organs	Histological findings	Group	1	4
		Dose (mg/kg)	0	2000
		No. of animals	5	5
Liver				
	no remarkable change		5	5
Glandular stomach				
	no remarkable change		5	4
	inflammation, focal		0	1

Table 18 Summary of Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4228 Test facility : Food and Drug Safety Center, Hatano Research Institute

		Group	1	4
		Dose (mg/kg)	0	62.5
Organs	Histological findings	No. of animals	5	5
Liver				
	no remarkable change		5	5
Glandular stomach				
	no remarkable change		5	5

Table 19 Summary of Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4229 Test facility : Food and Drug Safety Center, Hatano Research Institute

		Group	4
		Dose (mg/kg)	62.5
Organs	Histological findings	No. of animals	5
Liver			
	no remarkable change		1
	decrease, glycogen, hepatocyte		4
	hypertrophy, sinusoidal cell		1
Glandular stomach			
	no remarkable change		5

Table 20 Summary of Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4230 Test facility : Food and Drug Safety Center, Hatano Research Institute

		Group	4
		Dose (mg/kg)	2000
Organs	Histological findings	No. of animals	5
Liver			
	no remarkable change		5
Glandular stomach			
	no remarkable change		5

Table 21 Summary of Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4231 Test facility : Mitsubishi Chemical Medience Corp.

		Group	1	4
		Dose (mg/kg)	0	100
Organs	Histological findings	No. of animals	5	5
Liver				
	no remarkable change		4	0
	decrease, glycogen, hepatocyte		0	2
	vacuolation, hepatocyte, perilobular		0	3
	single cell necrosis, hepatocyte, centrilobular		0	4
	necrosis, hepatocyte, centrlilobular		0	1
	necrosis, hepatocyte, focal		1	1
	anisonucleosis, hepatocyte		0	5
	increase in mitosis, hepatocyte		0	2
Glandular stomach				
	no remarkable change		5	4
	single cell necrosis, chief cell		0	1

Table 22 Summary of Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4232 Test facility : Mitsubishi Chemical Medience Corp.

		Group	1	3
		Dose (mg/kg)	0	500
Organs	Histological findings	No. of animals	5	5
Liver				
	no remarkable change		5	0
	decrease, glycogen, hepatocyte		0	4
	erythrophagia, Kupffer cell		0	2
	necrosis, hepatocyte, focal		0	1
	hypertrophy, hepatocyte, perilobular		0	5
	increase in mitosis, hepatocyte		0	3
Glandular stomach				
	no remarkable change		5	5

Table 23 Summary of Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4233 Test facility : Mitsubishi Chemical Medience Corp.

		Group	1	2
		Dose (mg/kg)	0	2000
Organs	Histological findings	No. of animals	5	5
Liver				
	no remarkable change		4	0
	decrease, glycogen, hepatocyte		0	5
	eosinophilic body, hepatocyte		1	0
	single cell necrosis, hepatocyte		1	0
	hypertrophy, hepatocyte, perilobular		0	5
Glandular stomach				
	no remarkable change		5	5

Table 24 Summary of histological findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4234 Test facility : Sumitomo Chemical Company Limited

		Group	1	4
		Dose (mg/kg)	0	2000
Organs	Histological findings	No. of animals	5	5
Liver				
	no remarkable change		4	5
	necrosis, hepatocyte, focal		1	0
Glandular stomach				
	no remarkable change		5	5

Table 25 Summary of histological findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4235 Test facility : Sumitomo Chemical Company Limited

		Group	1	4
		Dose (mg/kg)	0	2000
Organs	Histological findings	No. of animals	5	5
Liver				
	no remarkable change		5	0
	decrease, glycogen, hepatocyte		0	5
	hypertrophy, hepatocyte, diffuse		0	2
	increase in mitosis, hepatocyte		0	5
Glandular stomach				
	no remarkable change		5	5

Table 26 Summary of histological findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4236 Test facility : Sumitomo Chemical Company Limited

		Group	1	4
		Dose (mg/kg)	0	2000
Organs	Histological findings	No. of animals	5	5
Liver				
	no remarkable change		5	1
	decrease, glycogen, hepatocyte		0	4
Glandular stomach				
	no remarkable change		5	5

Table 27 Summary of Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4237 Test facility : The Institute of Environmental Toxicology

Organs	Histological findings	Group	1	3	4
		Dose (mg/kg)	0	40	80
		No. of animals	5	5	5
Liver					
	no remarkable change		3	-	0
	decrease, glycogen, hepatocyte		0	-	5
	necrosis, hepatocyte, focal		1	-	0
	fibrosis, focal		2	-	0
Glandular stomach					
	no remarkable change		5	5	5

-: Not applicable

Table 28 Summary of Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4238 Test facility : The Institute of Environmental Toxicology

		Group	1	4
		Dose (mg/kg)	0	500
Organs	Histological findings	No. of animals	5	5
Liver				
	no remarkable change		5	0
	hemorrhage, centrilobular		0	1
	decrease, glycogen, hepatocyte		0	3
	degeneration, granular, hepatocyte		0	5
	single cell necrosis, hepatocyte, centrilobular		0	1
	necrosis, hepatocyte, centrilobular		0	1
	increase in mitosis, hepatocyte		0	1
	infiltration, inflammatory cell		0	5
Glandular stomach				
	no remarkable change		5	4
	necrosis, focal		0	1

Table 29 Summary of Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4239 Test facility : The Institute of Environmental Toxicology

		Group	1	4
		Dose (mg/kg)	0	2000
Organs	Histological findings	No. of animals	5	5
Liver				
	no remarkable change		5	0
	decrease, glycogen, hepatocyte		0	2
	shrinkage, hepatocyte		0	5
	anisonucleosis, hepatocyte		0	3
	increase in mitosis, hepatocyte		0	5
Glandular stomach				
	no remarkable change		5	3
	erosion		0	1
	necrosis, focal		0	1

Exp. No. D807 (079-519)

Table 30 Summary of histrogilcal finding and results of Comet assay

Organ: liver

Histological findings	Test substance (No. of animal/totall)		
	Results of Comet assay		
	Negative	Equivocal	Positive
anisonucleosis, hepatocyte	A4211 (3/5) A4223 (3/5)	A4224-1 (5/5)	A4201 (5/5) A4224-2 (4/5) A4231 (5/5)
anisonucleosis	A4239 (3/5)		
anisonucleosis, hepatocyte, perilobular	A4202 (5/5)		
decrease, glycogen, hepatocyte	A4202 (4/5) A4203 (2/5) A4212 (5/5) A4222 (2/3) A4223 (1/5) A4226 (1/5) A4229 (4/5) A4232 (4/5) A4233 (5/5) A4238 (3/5) A4239 (2/5)	A4219 (5/5) A4224-1 (5/5)	A4201 (5/5) A4210 (5/5) A4213 (1/5) A4214 (1/5) A4224-2 (5/5) A4231 (2/5) A4237 (5/5)
degeneration, vacuolar, hepatocyte, centrilobular	A4202 (5/5)		
degeneration, granular, hepatocyte	A4238 (5/5)		
erythrophagia, Kupper cell	A4232 (2/5)		
hemorrhage, centrilobular	A4238 (1/5)		
hypertrophy, hepatocyte, diffuse	A4212 (5/5)		
hypertrophy, hepatocyte, perilobular	A4202 (5/5) A4222 (3/3) A4232 (5/5) A4233 (5/5)	A4224-1 (3/5)	A4224-2 (3/5)
increase in mitosis, hepatocyte	A4211 (4/5) A4223 (3/5) A4226 (2/5) A4232 (3/5) A4239 (5/5) A4238 (1/5)		A4231 (2/5)
increase in mitosis, hepatocyte, perilobular	A4202 (4/5)		
infiltration, inflammatory cell	A4238 (5/5)		
infiltration, mononuclear cell	A4204 (4/5)	A4219 (1/5)	
necrosis, hepatocyte, focal	A4202 (2/5)		
necrosis, hepatocyte, centrilobular	A4238 (1/5)		A4231 (1/5)
shrinkage, nucleus, hepatocyte	A4212 (5/5)		
shrinkage, hepatocyte	A4239 (5/5)		
single cell necrosis, hepatocyte, centrilobular	A4202 (1/5) A4238 (1/5)	A4219 (1/5)	A4210 (5/5) A4231 (4/5)

Exp. No. D807 (079-519)

Table 30 -continued

Organ: liver

Histological findings	Test substance (No. of animal/totall)		
	Results of Comet assay		
	Negative	Equivocal	Positive
eosinophilic change, hepatocyte		A4224-1 (5/5)	A4224-2 (5/5)
hypertrophy, sinusoidal cell		A4224-1 (5/5)	A4224-2 (5/5)
nuclear enlargement, bile duct epithelium		A4219 (3/5)	
nuclear enlargement, hepatocyte		A4219 (5/5)	
proliferation, bile duct		A4224-1 (4/5)	A4224-2 (5/5)
single cell necrosis, hepatocyte		A4224-1 (5/5)	A4224-2 (5/5)
eosinophilic change, hepatocyte, perilobular			A4201(1/5)
infiltration, neutrophil, pericholangiolar			A4213 (5/5)
vacuolation, hepatocyte, perilobular			A4201(4/5) A4231 (3/5)
vacuolation, microvesicular, hepatocyte			A4210 (2/5)

Exp. No. D807 (079-519)

Table 30 -continued

Organ: stomach

Histological findings	Test substance (No. of animal/totall)		
	Results of Comet assay		
	Negative	Equivocal	Positive
edema, focal	A4224-2(1/5)		
single cell necrosis, chief cell	A4202 (2/5)		A4231 (1/5)
necrosis, focal	A4238 (1/5)		
erosion			A4213 (2/5)
proliferation, surface epithelial cell			A4213 (2/5)

Appendix 1 Individual Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4201 Test facility : BioReliance
Group : 1 Dose : 0 mg/kg

Animal ID No.	Organs	Histological findings
1	Liver	no remarkable change
	Glandular stomach	no remarkable change
2	Liver	no remarkable change
	Glandular stomach	no remarkable change
3	Liver	no remarkable change
	Glandular stomach	no remarkable change
4	Liver	no remarkable change
	Glandular stomach	no remarkable change
5	Liver	no remarkable change
	Glandular stomach	no remarkable change

Appendix 1 -continued *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4201 Test facility : BioReliance
Group : 4 Dose : 200 mg/kg

Animal ID No.	Organs	Histological findings
16	Liver	decrease, glycogen, hepatocyte anisonucleosis, hepatocyte
	Glandular stomach	no remarkable change
17	Liver	decrease, glycogen, hepatocyte vacuolation, hepatocyte, perilobular eosinophilic change, hepatocyte, perilobular anisonucleosis, hepatocyte
	Glandular stomach	no remarkable change
18	Liver	decrease, glycogen, hepatocyte vacuolation, hepatocyte, perilobular anisonucleosis, hepatocyte
	Glandular stomach	no remarkable change
19	Liver	decrease, glycogen, hepatocyte vacuolation, hepatocyte, perilobular anisonucleosis, hepatocyte
	Glandular stomach	no remarkable change
20	Liver	decrease, glycogen, hepatocyte vacuolation, hepatocyte, perilobular anisonucleosis, hepatocyte
	Glandular stomach	no remarkable change

Appendix 2 Individual Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4202 Test facility : BioReliance
Group : 1 Dose : 0 mg/kg

Animal ID No.	Organs	Histological findings
101	Liver	no remarkable change
	Glandular stomach	no remarkable change
102	Liver	no remarkable change
	Glandular stomach	no remarkable change
103	Liver	no remarkable change
	Glandular stomach	no remarkable change
104	Liver	no remarkable change
	Glandular stomach	no remarkable change
105	Liver	increase in mitosis, hepatocyte, panlobular
	Glandular stomach	no remarkable change

Appendix 2 -continued *Exp. No. D807 (079-519)*

Test substance : A4202 Test facility : BioReliance
Group : 4 Dose : 500 mg/kg

Animal ID No.	Organs	Histological findings
116	Liver	decrease, glycogen, hepatocyte degeneration, vacuolar, hepatocyte, centrilobular hypertrophy, hepatocyte, perilobular increase in mitosis, hepatocyte, perilobular anisonucleosis, hepatocyte, perilobular
	Glandular stomach	single cell necrosis, chief cell
117	Liver	decrease, glycogen, hepatocyte degeneration, vacuolar, hepatocyte, centrilobular hypertrophy, hepatocyte, perilobular necrosis, hepatocyte, focal with infiltration, neutrophil anisonucleosis, hepatocyte, perilobular
	Glandular stomach	no remarkable change
118	Liver	degeneration, vacuolar, hepatocyte, centrilobular, moderate hypertrophy, hepatocyte, perilobular necrosis, hepatocyte, focal with infiltration, neutrophil increase in mitosis, hepatocyte, perilobular anisonucleosis, hepatocyte, perilobular
	Glandular stomach	no remarkable change

Appendix 2 -continued *Exp. No. D807 (079-519)*

Test substance : A4202 Test facility : BioReliance
Group : 4 Dose : 500 mg/kg

Animal ID No.	Organs	Histological findings
119	Liver	decrease, glycogen, hepatocyte degeneration, vacuolar, hepatocyte, centrilobular, moderate hypertrophy, hepatocyte, perilobular increase in mitosis, hepatocyte, perilobular anisonucleosis, hepatocyte, perilobular single cell necrosis, hepatocyte, centrilobular with infiltration, neutrophil
	Glandular stomach	single cell necrosis, chief cell
120	Liver	degeneration, vacuolar, hepatocyte, centrilobular hypertrophy, hepatocyte, perilobular increase in mitosis, hepatocyte, perilobular anisonucleosis, hepatocyte, perilobular
	Glandular stomach	no remarkable change

Appendix 3 Individual Histological Findings *Exp. No. D807 (079-519)*

Test substance : A4203 Test facility : BioReliance
Group : 1 Dose : 0 mg/kg

Animal ID No.	Organs	Histological findings
1	Liver	no remarkable change
	Glandular stomach	no remarkable change
2	Liver	no remarkable change
	Glandular stomach	no remarkable change
3	Liver	no remarkable change
	Glandular stomach	no remarkable change
4	Liver	no remarkable change
	Glandular stomach	no remarkable change
5	Liver	no remarkable change
	Glandular stomach	no remarkable change

Appendix 3 -continued *Exp. No. D807 (079-519)*

Test substance : A4203 Test facility : BioReliance
Group : 4 Dose : 40 mg/kg

Animal ID No.	Organs	Histological findings
16	Liver	decrease, glycogen, hepatocyte
	Glandular stomach	no remarkable change
17	Liver	no remarkable change
	Glandular stomach	no remarkable change
18	Liver	no remarkable change
	Glandular stomach	no remarkable change
19	Liver	decrease, glycogen, hepatocyte
	Glandular stomach	no remarkable change
20	Liver	no remarkable change
	Glandular stomach	no remarkable change

Appendix 4 Individual Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4204 Test facility : ILS Inc.
Group : 1 Dose : 0 mg/kg

Animal ID No.	Organs	Histological findings
1	Liver	capsulitis, focal
	Glandular stomach	no remarkable change
2	Liver	no remarkable change
	Glandular stomach	no remarkable change
3	Liver	no remarkable change
	Glandular stomach	no remarkable change
4	Liver	no remarkable change
	Glandular stomach	no remarkable change
5	Liver	no remarkable change
	Glandular stomach	no remarkable change

Appendix 4 -continued *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4204 Test facility : ILS Inc.
Group : 4 Dose : 2.5 mg/kg

Animal ID No.	Organs	Histological findings
16	Liver	no remarkable change
	Glandular stomach	no remarkable change
17	Liver	no remarkable change
	Glandular stomach	no remarkable change
18	Liver	no remarkable change
	Glandular stomach	no remarkable change
19	Liver	no remarkable change
	Glandular stomach	no remarkable change
20	Liver	no remarkable change
	Glandular stomach	no remarkable change

Appendix 5 Individual Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4206 Test facility : ILS Inc.
Group : 1 Dose : 0 mg/kg

Animal ID No.	Organs	Histological findings
1	Liver	no remarkable change
	Glandular stomach	no remarkable change
2	Liver	no remarkable change
	Glandular stomach	no remarkable change
3	Liver	single cell necrosis, hepatocyte, focal
	Glandular stomach	no remarkable change
4	Liver	no remarkable change
	Glandular stomach	no remarkable change
5	Liver	no remarkable change
	Glandular stomach	no remarkable change

Appendix 5 -continued *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4206 Test facility : ILS Inc.
Group : 4 Dose : 6.25 mg/kg

Animal ID No.	Organs	Histological findings
16	Liver	no remarkable change
	Glandular stomach	no remarkable change
17	Liver	necrosis, hepatocyte, focal
	Glandular stomach	no remarkable change
18	Liver	no remarkable change
	Glandular stomach	no remarkable change
19	Liver	no remarkable change
	Glandular stomach	no remarkable change
20	Liver	no remarkable change
	Glandular stomach	no remarkable change

Appendix 6 Individual Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4210 Test facility : Huntingdon Life Science
Group : 4 Dose : 150 mg/kg

Animal ID No.	Organs	Histological findings
531	Liver	decrease, glycogen, hepatocyte single cell necrosis, hepatocyte, centrilobular
	Glandular Stomach	no remarkable change
532	Liver	decrease, glycogen, hepatocyte single cell necrosis, hepatocyte, centrilobular
	Glandular Stomach	no remarkable change
533	Liver	decrease, glycogen, hepatocyte single cell necrosis, hepatocyte, centrilobular
	Glandular Stomach	no remarkable change
534	Liver	decrease, glycogen, hepatocyte vacuolation, microvesicular, hepatocyte single cell necrosis, hepatocyte, centrilobular
	Glandular Stomach	no remarkable change
535	Liver	decrease, glycogen, hepatocyte vacuolation, microvesicular, hepatocyte single cell necrosis, hepatocyte, centrilobular
	Glandular Stomach	no remarkable change

Appendix 7 Individual Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4211 Test facility : Huntingdon Life Science
Group : 4 Dose : 525 mg/kg

Animal ID No.	Organs	Histological findings
631	Liver	increase in mitosis, hepatocyte
	Glandular Stomach	no remarkable change
632	Liver	increase in mitosis, hepatocyte anisonucleosis, hepatocyte
	Glandular Stomach	no remarkable change
633	Liver	increase in mitosis, hepatocyte anisonucleosis, hepatocyte
	Glandular Stomach	no remarkable change
635	Liver	no remarkable change
	Glandular Stomach	no remarkable change
651	Liver	increase in mitosis, hepatocyte, moderate anisonucleosis, hepatocyte
	Glandular Stomach	no remarkable change

Appendix 8 Individual Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4212 Test facility : Huntingdon Life Science
Group : 4 Dose : 2000 mg/kg

Animal ID No.	Organs	Histological findings
731	Liver	decrease, glycogen, hepatocyte shrinkage nucleus, hepatocyte hypertrophy, hepatocyte, diffuse
	Glandular Stomach	no remarkable change
732	Liver	decrease, glycogen, hepatocyte shrinkage nucleus, hepatocyte, moderate hypertrophy, hepatocyte, diffuse
	Glandular Stomach	no remarkable change
733	Liver	decrease, glycogen, hepatocyte shrinkage nucleus, hepatocyte, moderate hypertrophy, hepatocyte, diffuse
	Glandular Stomach	no remarkable change

Appendix 8 -continued *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4212 Test facility : Huntingdon Life Science
Group : 4 Dose : 2000 mg/kg

Animal ID No.	Organs	Histological findings
734	Liver	decrease, glycogen, hepatocyte shrinkage nucleus, hepatocyte hypertrophy, hepatocyte, diffuse
	Glandular Stomach	no remarkable change
735	Liver	decrease, glycogen, hepatocyte shrinkage nucleus, hepatocyte, moderate hypertrophy, hepatocyte, diffuse
	Glandular Stomach	no remarkable change

Appendix 9 Individual Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4213 Test facility : Novartis Pharmaceuticals Corporation
Group : 1 Dose : 0 mg/kg

Animal ID No.	Organs	Histological findings
1M	Liver	no remarkable change
	Glandular stomach	no remarkable change
2M	Liver	no remarkable change
	Glandular stomach	no remarkable change
3M	Liver	no remarkable change
	Glandular stomach	no remarkable change
4M	Liver	no remarkable change
	Glandular stomach	no remarkable change
5M	Liver	no remarkable change
	Glandular stomach	no remarkable change

Appendix 9 -continued *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4213 Test facility : Novartis Pharmaceuticals Corporation
Group : 4 Dose : 80 mg/kg

Animal ID No.	Organs	Histological findings
16M	Liver	infiltration, neutrophil, pericholangiolar
	Glandular stomach	erosion
		proliferation, surface epithelial cell
17M	Liver	infiltration, neutrophil, pericholangiolar
	Glandular stomach	erosion
18M	Liver	infiltration, neutrophil, pericholangiolar
	Glandular stomach	proliferation, surface epithelial cell
19M	Liver	infiltration, neutrophil, pericholangiolar
	Glandular stomach	no remarkable change
20M	Liver	infiltration, neutrophil, pericholangiolar
	Glandular stomach	no remarkable change

Appendix 10 Individual Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4214 Test facility : Novartis Pharmaceuticals Corporation
Group : 1 Dose : 0 mg/kg

Animal ID No.	Organs	Histological findings
1M1	Liver	no remarkable change
	Glandular stomach	no remarkable change
2M1	Liver	no remarkable change
	Glandular stomach	no remarkable change
3M1	Liver	no remarkable change
	Glandular stomach	no remarkable change
4M1	Liver	no remarkable change
	Glandular stomach	no remarkable change
5M1	Liver	no remarkable change
	Glandular stomach	no remarkable change

Appendix 10 -continued *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4214 Test facility : Novartis Pharmaceuticals Corporation
Group : 4 Dose : 600 mg/kg

Animal ID No.	Organs	Histological findings
16M1	Liver	no remarkable change
	Glandular stomach	no remarkable change
17M1	Liver	decrease, glycogen, hepatocyte
	Glandular stomach	no remarkable change
18M1	Liver	no remarkable change
	Glandular stomach	no remarkable change
19M1	Liver	no remarkable change
	Glandular stomach	no remarkable change
20M1	Liver	no remarkable change
	Glandular stomach	no remarkable change

Appendix 11 Individual Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4215 Test facility : Novartis Pharmaceuticals Corporation
Group : 1 Dose : 0 mg/kg

Animal ID No.	Organs	Histological findings
1M	Liver	no remarkable change
	Glandular stomach	no remarkable change
2M	Liver	no remarkable change
	Glandular stomach	no remarkable change
3M	Liver	no remarkable change
	Glandular stomach	no remarkable change
4M	Liver	no remarkable change
	Glandular stomach	no remarkable change
5M	Liver	no remarkable change
	Glandular stomach	no remarkable change

Appendix 11 -continued *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4215 Test facility : Novartis Pharmaceuticals Corporation
Group : 4 Dose : 100 mg/kg

Animal ID No.	Organs	Histological findings
16M	Liver	no remarkable change
	Glandular stomach	no remarkable change
17M	Liver	no remarkable change
	Glandular stomach	no remarkable change
18M	Liver	no remarkable change
	Glandular stomach	no remarkable change
19M	Liver	no remarkable change
	Glandular stomach	no remarkable change
20M	Liver	no remarkable change
	Glandular stomach	single cell necrosis

Appendix 12 Individual Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4219 Test facility : ILS Inc.
Group : 1 Dose : 0 mg/kg

Animal ID No.	Organs	Histological findings
1	Liver	no remarkable change
	Glandular stomach	no remarkable change
2	Liver	no remarkable change
	Glandular stomach	no remarkable change
3	Liver	no remarkable change
	Glandular stomach	no remarkable change
4	Liver	no remarkable change
	Glandular stomach	no remarkable change
5	Liver	no remarkable change
	Glandular stomach	no remarkable change

Appendix 12 -continued *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4219 Test facility : ILS Inc.
Group : 4 Dose : 30.0 mg/kg

Animal ID No.	Organs	Histological findings
16	Liver	decrease, glycogen, hepatocyte nuclear enlargement, hepatocyte
	Glandular stomach	no remarkable change
17	Liver	decrease, glycogen, hepatocyte nuclear enlargement, hepatocyte
	Glandular stomach	no remarkable change
18	Liver	decrease, glycogen, hepatocyte nuclear enlargement, hepatocyte nuclear enlargement, bile duct epithelium single cell necrosis, hepatocyte, centrilobular
	Glandular stomach	single cell necrosis
19	Liver	decrease, glycogen, hepatocyte nuclear enlargement, hepatocyte nuclear enlargement, bile duct epithelium
	Glandular stomach	no remarkable change

Appendix 12 -continued *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4219 Test facility : ILS Inc.
Group : 4 Dose : 30.0 mg/kg

Animal ID No.	Organs	Histological findings
20	Liver	decrease, glycogen, hepatocyte nuclear enlargement, hepatocyte nuclear enlargement, bile duct epithelium
	Glandular stomach	no remarkable change

Appendix 13 Individual Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4222 Test facility : Janssen Pharmaceuticals, Inc.
Group : 1 Dose : 0 mg/kg

Animal ID No.	Organs	Histological findings
1	Liver	no remarkable change
	Glandular stomach	no remarkable change
2	Liver	no remarkable change
	Glandular stomach	no remarkable change
3	Liver	no remarkable change
	Glandular stomach	no remarkable change
4	Liver	no remarkable change
	Glandular stomach	no remarkable change
5	Liver	no remarkable change
	Glandular stomach	no remarkable change

Appendix 13 -continued *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4222 Test facility : Janssen Pharmaceuticals, Inc.
Group : 4 Dose : 100 mg/kg

Animal ID No.	Organs	Histological findings
16	Liver	hypertrophy, hepatocyte, perilobular
	Glandular stomach	no remarkable change
17	Liver	decrease, glycogen, hepatocyte
		hypertrophy, hepatocyte, perilobular, moderate
	Glandular stomach	no remarkable change
19	Liver	decrease, glycogen, hepatocyte
		hypertrophy, hepatocyte, perilobular, moderate
	Glandular stomach	no remarkable change

Appendix 14 Individual Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4223 Test facility : Janssen Pharmaceuticals, Inc.
Group : 1 Dose : 0 mg/kg

Animal ID No.	Organs	Histological findings
1	Liver	no remarkable change
	Glandular stomach	no remarkable change
2	Liver	necrosis, hepatocyte, focal with infiltration, mononuclear cell
	Glandular stomach	no remarkable change
3	Liver	no remarkable change
	Glandular stomach	no remarkable change
4	Liver	no remarkable change
	Glandular stomach	no remarkable change
5	Liver	no remarkable change
	Glandular stomach	no remarkable change

Appendix 14 -continued *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4223 Test facility : Janssen Pharmaceuticals, Inc.
Group : 4 Dose : 1000 mg/kg

Animal ID No.	Organs	Histological findings
31	Liver	increase in mitosis, hepatocyte
	Glandular stomach	no remarkable change
32	Liver	decrease, glycogen, hepatocyte anisonucleosis, hepatocyte, moderate increase in mitosis, hepatocyte, moderate
	Glandular stomach	no remarkable change
33	Liver	no remarkable change
	Glandular stomach	no remarkable change
34	Liver	increase in mitosis, hepatocyte anisonucleosis, hepatocyte
	Glandular stomach	no remarkable change
35	Liver	anisonucleosis, hepatocyte
	Glandular stomach	no remarkable change

Appendix 15-1 Individual Histological Findings *Exp. No. D807 (079-519)*

Test substance : A4224 (first study) Test facility : Janssen Pharmaceuticals, Inc.
Group : 1 Dose : 0 mg/kg

Animal ID No.	Organs	Histological findings
1	Liver	no remarkable change
	Glandular stomach	no remarkable change
2	Liver	no remarkable change
	Glandular stomach	no remarkable change
3	Liver	no remarkable change
	Glandular stomach	no remarkable change
4	Liver	no remarkable change
	Glandular stomach	no remarkable change
5	Liver	no remarkable change
	Glandular stomach	no remarkable change

Appendix 15-1 -continued *Exp. No. D807 (079-519)*

Test substance : A4224 (first study) Test facility : Janssen Pharmaceuticals, Inc.
Group : 4 Dose : 150 mg/kg

Animal ID No.	Organs	Histological findings
31	Liver	decrease, glycogen, hepatocyte eosinophilic change, hepatocyte, moderate single cell necrosis, hepatocyte, scattered hypertrophy, hepatocyte, perilobular, moderate hypertrophy, sinusoidal cell anisonucleosis, hepatocyte
	Glandular stomach	no remarkable change
32	Liver	decrease, glycogen, hepatocyte eosinophilic change, hepatocyte, moderate single cell necrosis, hepatocyte, moderate, scattered hypertrophy, hepatocyte, perilobular hypertrophy, sinusoidal cell anisonucleosis, hepatocyte proliferation, bile duct
	Glandular stomach	no remarkable change
33	Liver	decrease, glycogen, hepatocyte eosinophilic change, hepatocyte single cell necrosis, hepatocyte, scattered hypertrophy, sinusoidal cell anisonucleosis, hepatocyte proliferation, bile duct
	Glandular stomach	no remarkable change

Appendix 15-1 -continued *Exp. No. D807 (079-519)*

Test substance : A4224 (first study) Test facility : Janssen Pharmaceuticals, Inc.
Group : 4 Dose : 150 mg/kg

Animal ID No.	Organs	Histological findings
34	Liver	decrease, glycogen, hepatocyte eosinophilic change, hepatocyte single cell necrosis, hepatocyte, marked, scattered hypertrophy, sinusoidal cell anisonucleosis, hepatocyte proliferation, bile duct
	Glandular stomach	no remarkable change
35	Liver	decrease, glycogen, hepatocyte eosinophilic change, hepatocyte single cell necrosis, hepatocyte, scattered hypertrophy, hepatocyte, perilobular hypertrophy, sinusoidal cell anisonucleosis, hepatocyte proliferation, bile duct fibrosis, focal
	Glandular stomach	no remarkable change

Appendix 15-2 Individual Histological Findings *Exp. No. D807 (079-519)*

Test substance : A4224 (second study) Test facility : Janssen Pharmaceuticals, Inc.
Group : 1 Dose : 0 mg/kg

Animal ID No.	Organs	Histological findings
1	Liver	no remarkable change
	Glandular stomach	no remarkable change
2	Liver	no remarkable change
	Glandular stomach	no remarkable change
3	Liver	no remarkable change
	Glandular stomach	no remarkable change
4	Liver	no remarkable change
	Glandular stomach	no remarkable change
5	Liver	no remarkable change
	Glandular stomach	no remarkable change

Appendix 15-2 -continued *Exp. No. D807 (079-519)*

Test substance : A4224 (second study) Test facility : Janssen Pharmaceuticals, Inc.
Group : 4 Dose : 200 mg/kg

Animal ID No.	Organs	Histological findings
31	Liver	decrease, glycogen, hepatocyte eosinophilic change, hepatocyte, moderate single cell necrosis, hepatocyte, marked, scattered hypertrophy, hepatocyte, perilobular hypertrophy, sinusoidal cell, moderate anisonucleosis, hepatocyte proliferation, bile duct
	Glandular stomach	no remarkable change
32	Liver	decrease, glycogen, hepatocyte eosinophilic change, hepatocyte, moderate single cell necrosis, hepatocyte, moderate, scattered hypertrophy, hepatocyte, perilobular hypertrophy, sinusoidal cell anisonucleosis, hepatocyte proliferation, bile duct, moderate
	Glandular stomach	no remarkable change

Appendix 15-2 -continued *Exp. No. D807 (079-519)*

Test substance : A4224 (second study) Test facility : Janssen Pharmaceuticals, Inc.
Group : 4 Dose : 200 mg/kg

Animal ID No.	Organs	Histological findings
33	Liver	decrease, glycogen, hepatocyte eosinophilic change, hepatocyte, moderate single cell necrosis, hepatocyte, marked, scattered hypertrophy, hepatocyte, perilobular hypertrophy, sinusoidal cell anisonucleosis, hepatocyte proliferation, bile duct
	Glandular stomach	edema, focal, superficial
34	Liver	decrease, glycogen, hepatocyte eosinophilic change, hepatocyte single cell necrosis, hepatocyte, marked, scattered hypertrophy, sinusoidal cell anisonucleosis, hepatocyte proliferation, bile duct
	Glandular stomach	no remarkable change

Appendix 15-2 -continued *Exp. No. D807 (079-519)*

Test substance : A4224 (second study) Test facility : Janssen Pharmaceuticals, Inc.
Group : 4 Dose : 200 mg/kg

Animal ID No.	Organs	Histological findings
35	Liver	decrease, glycogen, hepatocyte eosinophilic change, hepatocyte single cell necrosis, hepatocyte, sporadic hypertrophy, sinusoidal cell anisonucleosis, hepatocyte proliferation, bile duct
	Glandular stomach	no remarkable change

Appendix 16 Individual Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4226 Test facility : ILS Inc.
Group : 1 Dose : 0 mg/kg

Animal ID No.	Organs	Histological findings
1	Liver	no remarkable change
	Glandular stomach	no remarkable change
2	Liver	no remarkable change
	Glandular stomach	no remarkable change
3	Liver	no remarkable change
	Glandular stomach	no remarkable change
4	Liver	no remarkable change
	Glandular stomach	no remarkable change
5	Liver	no remarkable change
	Glandular stomach	no remarkable change

Appendix 16 -continued *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4226 Test facility : ILS Inc.
Group : 4 Dose : 600 mg/kg

Animal ID No.	Organs	Histological findings
16	Liver	no remarkable change
	Glandular stomach	no remarkable change
17	Liver	decrease, glycogen, hepatocyte
	Glandular stomach	no remarkable change
18	Liver	no remarkable change
	Glandular stomach	no remarkable change
19	Liver	increase in mitosis, hepatocyte
	Glandular stomach	no remarkable change
20	Liver	increase in mitosis, hepatocyte
	Glandular stomach	no remarkable change

Appendix 17 Individual Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4227 Test facility : ILS Inc.
Group : 1 Dose : 0 mg/kg

Animal ID No.	Organs	Histological findings
1	Liver	no remarkable change
	Glandular stomach	no remarkable change
2	Liver	no remarkable change
	Glandular stomach	no remarkable change
3	Liver	no remarkable change
	Glandular stomach	no remarkable change
4	Liver	no remarkable change
	Glandular stomach	no remarkable change
5	Liver	no remarkable change
	Glandular stomach	no remarkable change

Appendix 17 -continued *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4227 Test facility : ILS Inc.
Group : 4 Dose : 2000 mg/kg

Animal ID No.	Organs	Histological findings
16	Liver	no remarkable change
	Glandular stomach	no remarkable change
17	Liver	no remarkable change
	Glandular stomach	no remarkable change
18	Liver	no remarkable change
	Glandular stomach	no remarkable change
19	Liver	no remarkable change
	Glandular stomach	no remarkable change
20	Liver	no remarkable change
	Glandular stomach	inflammation, focal, submucosa, muscle layer

Appendix 18 Individual Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4228 Test facility : Food and Drug Safety Center, Hatano Research Institute
Group : 1 Dose : 0 mg/kg

Animal ID No.	Organs	Histological findings
M1	Liver	no remarkable change
	Glandular stomach	no remarkable change
M2	Liver	no remarkable change
	Glandular stomach	no remarkable change
M3	Liver	no remarkable change
	Glandular stomach	no remarkable change
M4	Liver	no remarkable change
	Glandular stomach	no remarkable change
M5	Liver	no remarkable change
	Glandular stomach	no remarkable change

Appendix 18 -continued *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4228 Test facility : Food and Drug Safety Center, Hatano Research Institute
Group : 4 Dose : 62.5 mg/kg

Animal ID No.	Organs	Histological findings
M16	Liver	no remarkable change
	Glandular stomach	no remarkable change
M17	Liver	no remarkable change
	Glandular stomach	no remarkable change
M18	Liver	no remarkable change
	Glandular stomach	no remarkable change
M19	Liver	no remarkable change
	Glandular stomach	no remarkable change
M20	Liver	no remarkable change
	Glandular stomach	no remarkable change

Appendix 19 Individual Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4229 Test facility : Food and Drug Safety Center, Hatano Research Institute
Group : 4 Dose : 62.5 mg/kg

Animal ID No.	Organs	Histological findings
M16	Liver	decrease, glycogen, hepatocyte
	Glandular stomach	no remarkable change
M17	Liver	decrease, glycogen, hepatocyte hypertrophy, sinusoidal cell
	Glandular stomach	no remarkable change
M18	Liver	decrease, glycogen, hepatocyte
	Glandular stomach	no remarkable change
M19	Liver	no remarkable change
	Glandular stomach	no remarkable change
M20	Liver	decrease, glycogen, hepatocyte
	Glandular stomach	no remarkable change

Appendix 20 Individual Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4230 Test facility : Food and Drug Safety Center, Hatano Research Institute
Group : 4 Dose : 2000 mg/kg

Animal ID No.	Organs	Histological findings
M16	Liver	no remarkable change
	Glandular stomach	no remarkable change
M17	Liver	no remarkable change
	Glandular stomach	no remarkable change
M18	Liver	no remarkable change
	Glandular stomach	no remarkable change
M19	Liver	no remarkable change
	Glandular stomach	no remarkable change
M20	Liver	no remarkable change
	Glandular stomach	no remarkable change

Appendix 21 Individual Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4231 Test facility : Mitsubishi Chemical Medience Corp.
Group : 1 Dose : 0 mg/kg

Animal ID No.	Organs	Histological findings
10101	Liver	no remarkable change
	Glandular stomach	no remarkable change
10102	Liver	no remarkable change
	Glandular stomach	no remarkable change
10103	Liver	no remarkable change
	Glandular stomach	no remarkable change
10104	Liver	no remarkable change
	Glandular stomach	no remarkable change
10105	Liver	necrosis, hepatocyte, focal with infiltration, mononuclear cell
	Glandular stomach	no remarkable change

Appendix 21 -continued *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4231 Test facility : Mitsubishi Chemical Medience Corp.
Group : 4 Dose : 100 mg/kg

Animal ID No.	Organs	Histological findings
10401	Liver	decrease, glycogen, hepatocyte vacuolation, hepatocyte, perilobular anisonucleosis, hepatocyte single cell necrosis, hepatocyte, centrilobular with infiltration, mononuclear cell
	Glandular stomach	no remarkable change
10402	Liver	vacuolation, hepatocyte, perilobular anisonucleosis, hepatocyte single cell necrosis, hepatocyte, centrilobular with infiltration, mononuclear cell
	Glandular stomach	no remarkable change
10403	Liver	decrease, glycogen, hepatocyte anisonucleosis, hepatocyte necrosis, hepatocyte, centrilobular, marked
	Glandular stomach	single cell necrosis, chief cell
10404	Liver	vacuolation, hepatocyte, perilobular anisonucleosis, hepatocyte increase in mitosis, hepatocyte single cell necrosis, hepatocyte, centrilobular with infiltration, mononuclear cell
	Glandular stomach	no remarkable change

Appendix 21 -continued *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4231 Test facility : Mitsubishi Chemical Medience Corp.
Group : 4 Dose : 100 mg/kg

Animal ID No.	Organs	Histological findings
10405	Liver	anisonucleosis, hepatocyte increase in mitosis, hepatocyte single cell necrosis, hepatocyte, centrilobular with infiltration, mononuclear cell necrosis, hepatocyte, focal with infiltration, inflammatory cell
	Glandular stomach	no remarkable change

Appendix 22 Individual Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4232 Test facility : Mitsubishi Chemical Medience Corp.
Group : 1 Dose : 0 mg/kg

Animal ID No.	Organs	Histological findings
10101	Liver	no remarkable change
	Glandular stomach	no remarkable change
10102	Liver	no remarkable change
	Glandular stomach	no remarkable change
10103	Liver	no remarkable change
	Glandular stomach	no remarkable change
10104	Liver	no remarkable change
	Glandular stomach	no remarkable change
10105	Liver	no remarkable change
	Glandular stomach	no remarkable change

Appendix 22 -continued *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4232 Test facility : Mitsubishi Chemical Medience Corp.
Group : 3 Dose : 500 mg/kg

Animal ID No.	Organs	Histological findings
10501	Liver	decrease, glycogen, hepatocyte increase in mitosis, hepatocyte erythrophagia, Kupffer cell hypertrophy, hepatocyte, perilobular
	Glandular stomach	no remarkable change
10502	Liver	hypertrophy, hepatocyte, perilobular
	Glandular stomach	no remarkable change
10503	Liver	decrease, glycogen, hepatocyte hypertrophy, hepatocyte, perilobular increase in mitosis, hepatocyte
	Glandular stomach	no remarkable change
10504	Liver	decrease, glycogen, hepatocyte erythrophagia, Kupffer cell hypertrophy, hepatocyte, perilobular necrosis, hepatocyte, focal with infiltration, neutrophil
	Glandular stomach	no remarkable change

Appendix 22 -continued *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4232 Test facility : Mitsubishi Chemical Medience Corp.
Group : 3 Dose : 500 mg/kg

Animal ID No.	Organs	Histological findings
10505	Liver	decrease, glycogen, hepatocyte increase in mitosis, hepatocyte hypertrophy, hepatocyte, perilobular
	Glandular stomach	no remarkable change

Appendix 23 Individual Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4233 Test facility : Mitsubishi Chemical Medience Corp.
Group : 1 Dose : 0 mg/kg

Animal ID No.	Organs	Histological findings
10101	Liver	no remarkable change
	Glandular stomach	no remarkable change
10102	Liver	eosinophilic body, hepatocyte single cell necrosis, hepatocyte
	Glandular stomach	no remarkable change
10103	Liver	no remarkable change
	Glandular stomach	no remarkable change
10104	Liver	no remarkable change
	Glandular stomach	no remarkable change
10105	Liver	no remarkable change
	Glandular stomach	no remarkable change

Appendix 23 -continued *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4233 Test facility : Mitsubishi Chemical Medience Corp.
Group : 2 Dose : 2000 mg/kg

Animal ID No.	Organs	Histological findings
10401	Liver	decrease, glycogen, hepatocyte hypertrophy, hepatocyte, perilobular
	Glandular stomach	no remarkable change
10402	Liver	decrease, glycogen, hepatocyte hypertrophy, hepatocyte, perilobular
	Glandular stomach	no remarkable change
10403	Liver	decrease, glycogen, hepatocyte hypertrophy, hepatocyte, perilobular
	Glandular stomach	no remarkable change
10404	Liver	decrease, glycogen, hepatocyte hypertrophy, hepatocyte, perilobular
	Glandular stomach	no remarkable change
10405	Liver	decrease, glycogen, hepatocyte hypertrophy, hepatocyte, perilobular
	Glandular stomach	no remarkable change

Appendix 24 Individual Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4234 Test facility : Sumitomo Chemical Company Limited
Group : 1 Dose : 0 mg/kg

Animal ID No.	Organs	Histological findings
101	Liver	no remarkable change
	Glandular stomach	no remarkable change
102	Liver	no remarkable change
	Glandular stomach	no remarkable change
103	Liver	no remarkable change
	Glandular stomach	no remarkable change
104	Liver	necrosis, hepatocyte, focal with infiltration, inflammatory cell
	Glandular stomach	no remarkable change
105	Liver	no remarkable change
	Glandular stomach	no remarkable change

Appendix 24 -continued *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4234 Test facility : Sumitomo Chemical Company Limited
Group : 4 Dose : 2000 mg/kg

Animal ID No.	Organs	Histological findings
401	Liver	no remarkable change
	Glandular stomach	no remarkable change
402	Liver	no remarkable change
	Glandular stomach	no remarkable change
403	Liver	no remarkable change
	Glandular stomach	no remarkable change
404	Liver	no remarkable change
	Glandular stomach	no remarkable change
405	Liver	no remarkable change
	Glandular stomach	no remarkable change

Appendix 25 Individual Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4235 Test facility : Sumitomo Chemical Company Limited
Group : 1 Dose : 0 mg/kg

Animal ID No.	Organs	Histological findings
101	Liver	no remarkable change
	Glandular stomach	no remarkable change
102	Liver	no remarkable change
	Glandular stomach	no remarkable change
103	Liver	no remarkable change
	Glandular stomach	no remarkable change
104	Liver	no remarkable change
	Glandular stomach	no remarkable change
105	Liver	no remarkable change
	Glandular stomach	no remarkable change

Appendix 25 -continued *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4235 Test facility : Sumitomo Chemical Company Limited
Group : 4 Dose : 2000 mg/kg

Animal ID No.	Organs	Histological findings
401	Liver	decrease, glycogen, hepatocyte increase in mitosis, hepatocyte
	Glandular stomach	no remarkable change
402	Liver	decrease, glycogen, hepatocyte increase in mitosis, hepatocyte
	Glandular stomach	no remarkable change
403	Liver	decrease, glycogen, hepatocyte increase in mitosis, hepatocyte
	Glandular stomach	no remarkable change
404	Liver	decrease, glycogen, hepatocyte, moderate hypertrophy, hepatocyte, diffuse increase in mitosis, hepatocyte
	Glandular stomach	no remarkable change
405	Liver	decrease, glycogen, hepatocyte, moderate hypertrophy, hepatocyte, diffuse increase in mitosis, hepatocyte
	Glandular stomach	no remarkable change

Appendix 26 Individual Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4236 Test facility : Sumitomo Chemical Company Limited
Group : 1 Dose : 0 mg/kg

Animal ID No.	Organs	Histological findings
101	Liver	no remarkable change
	Glandular stomach	no remarkable change
102	Liver	no remarkable change
	Glandular stomach	no remarkable change
103	Liver	no remarkable change
	Glandular stomach	no remarkable change
104	Liver	no remarkable change
	Glandular stomach	no remarkable change
105	Liver	no remarkable change
	Glandular stomach	no remarkable change

Appendix 26 -continued *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4236 Test facility : Sumitomo Chemical Company Limited
Group : 4 Dose : 2000 mg/kg

Animal ID No.	Organs	Histological findings
401	Liver	no remarkable change
	Glandular stomach	no remarkable change
402	Liver	decrease, glycogen, hepatocyte
	Glandular stomach	no remarkable change
403	Liver	decrease, glycogen, hepatocyte
	Glandular stomach	no remarkable change
404	Liver	decrease, glycogen, hepatocyte
	Glandular stomach	no remarkable change
405	Liver	decrease, glycogen, hepatocyte
	Glandular stomach	no remarkable change

Appendix 27 Individual Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4237 Test facility : The Institute of Environmental Toxicology
Group : 1 Dose : 0 mg/kg

Animal ID No.	Organs	Histological findings
111	Liver	no remarkable change
	Glandular stomach	no remarkable change
112	Liver	no remarkable change
	Glandular stomach	no remarkable change
113	Liver	no remarkable change
	Glandular stomach	no remarkable change
114	Liver	fibrosis, focal with infiltration, mononuclear cell
	Glandular stomach	no remarkable change
115	Liver	necrosis, hepatocyte, focal with infiltration, mononuclear cell fibrosis, focal with infiltration, mononuclear cell, multiple
	Glandular stomach	no remarkable change

Appendix 27 -continued *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4237 Test facility : The Institute of Environmental Toxicology
Group : 3 Dose : 40 mg/kg

Animal ID No.	Organs	Histological findings
121	Liver	not examined
	Glandular stomach	no remarkable change
122	Liver	not examined
	Glandular stomach	no remarkable change
123	Liver	not examined
	Glandular stomach	no remarkable change
124	Liver	not examined
	Glandular stomach	no remarkable change
125	Liver	not examined
	Glandular stomach	no remarkable change

Appendix 27 -continued *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4237 Test facility : The Institute of Environmental Toxicology
Group : 4 Dose : 80 mg/kg

Animal ID No.	Organs	Histological findings
126	Liver	decrease, glycogen, hepatocyte
	Glandular stomach	no remarkable change
127	Liver	decrease, glycogen, hepatocyte
	Glandular stomach	no remarkable change
128	Liver	decrease, glycogen, hepatocyte
	Glandular stomach	no remarkable change
129	Liver	decrease, glycogen, hepatocyte
	Glandular stomach	no remarkable change
130	Liver	decrease, glycogen, hepatocyte
	Glandular stomach	no remarkable change

Appendix 28 Individual Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4238 Test facility : The Institute of Environmental Toxicology
Group : 1 Dose : 0 mg/kg

Animal ID No.	Organs	Histological findings
111	Liver	no remarkable change
	Glandular stomach	no remarkable change
112	Liver	no remarkable change
	Glandular stomach	no remarkable change
113	Liver	no remarkable change
	Glandular stomach	no remarkable change
114	Liver	no remarkable change
	Glandular stomach	no remarkable change
115	Liver	no remarkable change
	Glandular stomach	no remarkable change

Appendix 28 -continued *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4238 Test facility : The Institute of Environmental Toxicology
Group : 4 Dose : 500 mg/kg

Animal ID No.	Organs	Histological findings
126	Liver	degeneration, granular, hepatocyte, centrilobular, moderate increase in mitosis, hepatocyte infiltration, inflammatory cell, centrilobular
	Glandular stomach	no remarkable change
127	Liver	degeneration, granular, hepatocyte, centrilobular, moderate infiltration, inflammatory cell, centrilobular
	Glandular stomach	no remarkable change
128	Liver	decrease, glycogen, hepatocyte degeneration, granular, hepatocyte, centrilobular, marked infiltration, inflammatory cell, moderate, multifocal
	Glandular stomach	no remarkable change
129	Liver	decrease, glycogen, hepatocyte degeneration, granular, hepatocyte, centrilobular, marked single cell necrosis, hepatocyte, centrilobular infiltration, inflammatory cell, centrilobular
	Glandular stomach	no remarkable change

Appendix 28 -continued *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4238 Test facility : The Institute of Environmental Toxicology
Group : 4 Dose : 500 mg/kg

Animal ID No.	Organs	Histological findings
130	Liver	hemorrhage, centrilobular, moderate decrease, glycogen, hepatocyte degeneration, granular, hepatocyte, centrilobular, moderate necrosis, hepatocyte, centrilobular, moderate infiltration, inflammatory cell, centrilobular, marked
	Glandular stomach	necrosis, focal, mucosa, pyloric region

Appendix 29 Individual Histological Findings *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4239 Test facility : The Institute of Environmental Toxicology
Group : 1 Dose : 0 mg/kg

Animal ID No.	Organs	Histological findings
111	Liver	no remarkable change
	Glandular stomach	no remarkable change
112	Liver	no remarkable change
	Glandular stomach	no remarkable change
113	Liver	no remarkable change
	Glandular stomach	no remarkable change
114	Liver	no remarkable change
	Glandular stomach	no remarkable change
115	Liver	no remarkable change
	Glandular stomach	no remarkable change

Appendix 29 -continued *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4239 Test facility : The Institute of Environmental Toxicology
Group : 4 Dose : 2000 mg/kg

Animal ID No.	Organs	Histological findings
126	Liver	decrease, glycogen, hepatocyte shrinkage, hepatocyte, scattering anisonucleosis, hepatocyte increase in mitosis, hepatocyte
	Glandular stomach	no remarkable change
127	Liver	decrease, glycogen, hepatocyte shrinkage, hepatocyte, moderate, scattering anisonucleosis, hepatocyte increase in mitosis, hepatocyte
	Glandular stomach	necrosis, focal, pyloric region
128	Liver	shrinkage, hepatocyte, scattering increase in mitosis, hepatocyte
	Glandular stomach	erosion, pyloric region
129	Liver	shrinkage, hepatocyte, moderate, scattering increase in mitosis, hepatocyte
	Glandular stomach	no remarkable change

Appendix 29 -continued *Exp. No. D807 (079-519)*

Test substance (Code No.) : A4239 Test facility : The Institute of Environmental Toxicology
Group : 4 Dose : 2000 mg/kg

Animal ID No.	Organs	Histological findings
130	Liver	shrinkage, hepatocyte, marked, scattering anisonucleosis, hepatocyte increase in mitosis, hepatocyte
	Glandular stomach	no remarkable change

Addendum to the Comet Assay Validation Report

Expert Group Responses to the Peer Review Report

MAJOR RECOMMENDATIONS OF THE PEER REVIEW PANEL

The report of the JaCVAM initiative international validation studies of the *in vivo* rodent alkaline comet assay for the detection of genotoxic carcinogens was assessed by a Panel of experts from the OECD expert group for the development of the in vivo Comet assay. The peer review report is available as Document 197 in the Series on Testing and Assessment [ENV/JM/MONO(2014)11]. The main recommendations from the Peer Review Panel are reported below:

• Use of additional data including data from the literature in order to:

- address inter-laboratory reproducibility,

- broaden the applicability domain of the assay to classes of chemicals not included or not sufficiently represented in the validation exercise,

- broaden the scope of the TG to other species, gender, tissues.

- better describe the mechanism underpinning the assay, in particular the link between DNA migration observed in the assay and DNA damage,

- assess the advantage of the comet assay over the UDS assay,

• Analyse further some of the data from the validation study, to see if using the mean or the median for all the data can reduce the variations observed between laboratories and thus improve quantitative inter-laboratory reproducibility.

• Provide clearer guidance on methods of measurement of toxicity in the tissue being examined and on interpretation of test results when toxicity is found.

• Revise the regulatory purpose of the test, as it should not be used as the sole predictor for carcinogenicity.

• Recognise the limitations of the assay:

- some organs (such as the stomach) may lead to relatively high background of DNA fragmentation and high variability

- the assay has a low capability to reveal some type of damage without protocol adaptations

• Consider the need to develop some recommendations on how laboratory proficiency for tissues other than those tested in the validation study should be demonstrated.

RESULT OF THE DISCUSSION OF THE EXPERT GOUP ON THE COMET ASSAY

The Expert Group on the comet assay worked on the development of the draft TG between March 2013 and February 2014. During this period, a face to face meeting was held (Paris, 19-21 March 2013) as well as regular teleconferences to address the peer review comments and the comments received from two WNT rounds of comments organised in May and September 2013.

The objective of this document is to address the peer review comments based on the outcome of expert group discussions and/or further analyses. Most of these issues were addresses at the March meeting of the expert group.

Better describe the mechanism underpinning the assay, in particular the link between DNA migration observed in the assay and DNA damage

The expert group agreed that the mechanism underpinning the comet assay was appropriately described in the published literature. Thus the group agreed that this recommendation would be addressed in the Test Guideline itself by referring to a few publications describing the principles of the assay.

- Tice RR, Strauss GH., The single cell gel electrophoresis/Comet assay: a potential tool for detecting radiation-induced DNA damage in humans. Stem Cells 1995; 13 Suppl 1:207-14.

- Collins AR. The Comet assay for DNA damage and repair: principles, applications, and limitations. Mol Biotechnol. 2004; 26:249-61.

- Hartmann A, Schumacher M, Plappert-Helbig U, Lowe P, Suter W, Mueller L. Use of the alkaline in vivo Comet assay for mechanistic genotoxicity investigations. Mutagenesis 2004; 19:51-9.

Revise the regulatory purpose of the test, as it should not be used as the sole predictor for carcinogenicity

The group confirmed that the comet assay is an assay that measures DNA damages that manifest as strand breaks, but it should not be seen to be predictive only of carcinogenicity. Especially because the comet results alone do not indicate which types of modes of action led to the DNA damage nor does it inform on the various consequences of this DNA damage.

It was clarified that under alkaline conditions, the Comet assay can detect single and double stranded breaks, resulting, for example, from direct interactions with DNA, alkali labile sites or as a consequence of incomplete excision repair.

Assess the advantage of the comet assay over the UDS assay

The expert group didn't perform a detailed assessment of the advantage of the comet assay over the UDS assay but referred to a few papers in the literature which show the advantage of the comet assay over the UDS at least in terms of predicting carcinogenicity (Kirkland and Speit, 2008; OECD DRP 103, 2009). In addition, it was mentioned that another advantage of the Comet assay upon UDS assay was its ability to assay for genotoxic responses in a wide variety of tissues.

- Kirkland D, Speit G. Evaluation of the ability of a battery of three *in vitro* genotoxicity tests to discriminate rodent carcinogens and non-carcinogens III. Appropriate follow-up testing *in vivo*. Mutat. Res. 2008; 654:114-32.

- OECD (2009) Detailed Review Paper N° 103 on Transgenic Rodent Mutation Assays.

Address inter-laboratory reproducibility

One of the main concerns raised by the peer review report relates to the ability of the validation trial to demonstrate reproducibility. Because of the design utilized during the last phase of the validation trial, data for evaluating inter-laboratory reproducibility was limited mostly to positive and negative controls. For these chemicals, all participating laboratories obtained expected results from a qualitative (outcome) point of view but the magnitude of the effect was shown to be highly variable. Concerns were expressed that it is unclear how this significant variation, e.g. in the magnitude of the positive response among laboratories, would affect the detection of weak genotoxic agents by those laboratories with a weak positive control response.

The group acknowledged a high quantitative variability, but commented that such quantitative variability has been seen in many collaborative trials, even those using other test systems such as the Ames test. Therefore, in many ways the quantitative variability was not surprising. The group also recognised the inherent biological variability (animals sourced from different suppliers) and the strong influence of some experimental conditions (e.g. temperature, electrophoresis time). It was agreed that the TG will give guidance on factors that contribute to the variability, so that they can be controlled in order to reduce variability linked to the experimental condition. In addition to general guidance, the reader will be directed to published literature for details.

The group conducted a more thorough review of phases 4-1 and 4-2 of the validation trial in combination with the review of additional in house data and data from the public domain. The analysis presented at the meeting including graphs is available in the Annex 1 of this addendum.

Based on this review, most of the EG members considered the qualitative reproducibility to be good and the quantitative reproducibility to be acceptable. It was acknowledged that this reproducibility is based mainly on one potent positive chemical (although there was some evidence of reproducibility in the Rothfuss at al. (2010) paper for other chemicals). Although there was not full consensus on this issue it was agreed that it might not be justified to do more experimental testing for the demonstration of reproducibility/sensitivity of weak genotoxins.

The group also discussed how to identify critical factors for the evaluation of the assay. The analysis of the reproducibility of the assay in the JaCVAM trial shows (Figure 3 in Annex 1) some variability in the response to a single dose of EMS among laboratories. To have a better understanding of the heterogeneity of laboratory responses, the group had a look at the possible impact of the various parameters of the assay.

An Excel spread sheet was developed (Annex 2). It presents the values from the laboratories that took part in the JaCVAM trial for each important parameter of the assay and it enables an easy comparison of how these parameters might have affected the negative control values and positive control responses between laboratories. The objective of this task was to identify the critical variables i.e. those which variation would most likely explain between laboratory variability. The initial exercise focused on reporting critical variables for 4 laboratories that showed extreme (high and low)

responses with EMS in the JaCVAM trial. The work was further extended to the 13 laboratories that participated in the 1st step of 4th phase validation study.

It was noted that the JaCVAM protocol was quite prescriptive for many parameters except the preparation method of single cell suspensions (from necropsy to addition of the cells in the agarose to the slide, including the amount of agarose matrix added and the nature of the undercoating of the slide). One hypothesis is that these differences could introduce some variability in the results. But as the procedure is unevenly described in the laboratory reports, the analysis of the impact of these differences is limited.

For most variables it was not possible to show a real trend from the variations between labs. The most critical variables the group identified were the following:

- Voltage
- Current
- Electrophoresis time
- DNA unwinding time

The TG was revised in a way that enables the user to know what needs to be controlled but without being too prescriptive in terms of value recommendation.

Analyse further some of the data from the validation study, to see if using the mean or the median for all the data can reduce the variations observed between laboratories and thus improve quantitative inter-laboratory reproducibility

The group discussed if a more consistent use of statistical parameters could reduce the quantitative inter-laboratory variability.

In the original JaCVAM trial all data was calculated as means i.e. the mean value of the 50 cells/slide and the mean of the 3 slides was calculated giving a mean value per animal. The mean of the 5 animals per group was then calculated. However, analyses by a number of groups e.g. Bright et al. (2011), Lovell and Omori (2008), suggest that the median value would be more appropriate and would lead to a more sensitive analysis of the data.

Omori, the statistician who did the original analysis has kindly re-analysed the data from the 40 chemicals studied in part 4.2 of the JaCVAM trial and calculated the median values of the slides. The re-calculated data are available at the site given below:

https://webdisk.doshisha.ac.jp/public/ekbsgAuNawaA64MBUhxCMRpwQnM7XG_dbwdab8PIQvsM

The re-calculated data from Omori were reviewed by Brian Burlinson, an expert of the expert group on the comet assay and the statistical conclusion from the original table (Table 2 'Summary of 4th Phase-2nd Step Validation Study Results', in the report of the JACVAM international validation study) compared to the one produced by taking the mean of the medians. The seven pdf files listed below (available from the site given above) are those in which there has been a change in the statistical outcome – either from negative or equivocal to positive. There were no instances where the result went from positive to negative.

- A4211.pdf;
- A4219.pdf;
- A4220.pdf;

- A4226.pdf;
- A4228.pdf;
- A4229.pdf;
- A4237.pdf

A very simplistic view is that taking the median rather than the mean increases sensitivity, perhaps not surprising since the biggest change is on control value generally making it smaller; making increases due to test material more easily detected.

Need for transformation (e.g. log or square root) of the data

As a follow up, discussions on statistics were held with Simon Bates, an author of the paper developed by the Statisticians in the Pharmaceutical Industry (PSI) Toxicology Special Interest Group (Bright at al., 2011), focussing on as to when to do the log transformation i.e. add a small figure, 0.001, to all of the data to remove zeros then do a log transformation before calculating the median or calculating the median and then log transforming that figure. The outcome of these discussions is that it is unlikely that transformation make any difference. Adding offsets (while a commonly used and reliable technique) does suffer from the criticism of the choice of offset – it can, if the wrong choice of offset is made, influence the statistical results and hence is a bit arbitrary. The authors of the Bright et al. paper decided in this case to get around this (minor) criticism by taking the median of the 50 cells per slide first.

The only problem with this approach is if there is more that 50% zeros on a slide (highly unlikely though), then using the 0.001 offset is still a viable technique whereas the median = 0 and therefore can't be logged.

Simon Bates has tried many approaches (averaging the 150 cells and then logging, taking means of three logged slide medians, taking the median of the three logged slide means, adding offsets etc.) and all of them gave pretty much the same statistical results. The key thing was the need to log transform (at some point) prior to analysis.

In their paper Bright et al. (2011), suggest that if there are a lot of zeros (>25%) then it is worth calculating the median of the other non-zero values.

The log transformation of the data after adding a small number was also recommended by Smith et al. (2008), although in their paper they calculated the means of the slides.

Conclusion

In conclusion it would appear there are a range of views as to what is the appropriate measure but it would appear from the JaCVAM data and the view of a number of statisticians that calculating the median is preferable, followed by some transformation e.g. log or square root.

Recognise that the assay has a low capability to reveal some type of damage without protocol adaptations and broaden the applicability domain of the assay to classes of chemicals not included or not sufficiently represented in the validation exercise.

The group recognised that the current protocol is not aimed at the detection of DNA cross-links or oxidised bases. Specific detection of DNA cross-links or oxidised bases would require protocol adaptations. These modifications can be the induction of DNA damage with a known genotoxic agent or increase electrophoresis time in order to detect DNA cross-links or the addition of lesion-specific endonucleases to increase the sensitivity of the Comet assay to certain types of oxidised bases. It was noted that the assay however, can't be adapted for the detection of aneugens.

The section on Initial consideration and limitations in the Test Guideline includes reference to the published literature on how to adapt the protocol for detection of cross linking agents but such modified protocols are not covered by the Test Guideline.

Broaden the scope of the TG to other species, gender, tissues while considering the need to develop some recommendations on how laboratory proficiency for tissues other than those tested in the validation study should be demonstrated.

Based on the extensive literature available on the comet assay in various species and tissues, the group agreed in principle that the TG can be broadened to other species and tissues and not limited to only those evaluated in the validation trial. The scope of the assay was however limited to mammalian species (and the title revised accordingly) to suit regulatory purposes. It was clarified that the choice of the species and tissues needs justification and should be made based on all available data.

However, it was acknowledged that (i) the possibility to use tissues that had not been evaluated under the JaCVAM trial and (ii) the difference of background level among tissues, necessitate careful recommendation in terms of laboratory proficiency demonstration. The group agreed that initial efforts could focus on establishing proficiency with the most commonly used system i.e. the rodent liver. In addition, laboratories performing the test should establish negative control ranges for each tissue of interest, which should be low enough so as to allow for the detection of induced responses, and should be demonstrated to be reproducible.

In terms of choice of the gender in the comet assay, it was recognised that the number of studies performed to date on females is very limited and thus the recommendation to use males and/or female animal cannot be based on available data. Recognising that in general, other *in vivo* genotoxicity responses are similar between male and female animals, it was agreed to use for the comet assay the data driven approach used for the *in vivo* micronucleus assay, i.e. use of males only as the default, and use animals of both sexes in case there are insufficient data to establish lack of difference between the sexes.

Provide clearer guidance on methods of measurement of toxicity in the tissue being examined and on interpretation of test results when toxicity is found.

The expert group recognised that knowledge of the relationship between cytotoxicity and DNA damage is limited and it is not clear how to interpret genotoxicity data relative to cytotoxicity. Based on available knowledge of the various tools for measuring cytotoxicity (e.g. histopathology, hedgehogs, neutral diffusion), a few general recommendations were developed though, e.g.:

- An increase in the frequency of hedgehogs may indicate poor tissue quality.
- The presence of hedgehogs alone is not sufficient to consider that the DNA strand breaks, if seen, are not biologically relevant and could be a consequence of cytotoxicity.
- The neutral diffusion assay does not provide any useful information on cytotoxicity.
- The observation of mild to moderate histopathological changes (inflammation, infiltration, apoptotic or necrotic changes) associated with increased tail length of comets may not enable to distinguish whether these are due to genotoxic or cytotoxic effects.
- Dose levels inducing severe histopathological changes should be avoided. It may be necessary to extend the in-life observation period of the range-finder to see whether such severe effects lead to animal death or lack of tolerance.

References

Bright J, Aylott M, Bates S, Geys H, Jarvis P, Saul J and Vonk R (2011), Recommendations on the statistical analysis of the comet assay. Pharmaceutical Statistics, 10, 485-493

Lovell and Omori, 2008, Statistical issues in the use of the comet assay. Mutagenesis, 23, 171-182

Smith CC, Adkins DJ, Martin EA and O'Donovan MR (2008), Recommendations for the design of the rat comet assay. Mutagenesis, 23, 233-240

Annex 1: *In vivo* Comet assay: Additional comments on intra- and inter-laboratory reproducibility

(prepared by D J Kirkland, based on Data collected by B Burlinson, P Escobar and M Guerard)

In the 3rd phase pre-validation and 4th phase validation stages of the JaCVAM collaborative trial of the *in vivo* Comet assay, i.e. at stages where a robust protocol had been developed, the intra- and inter-laboratory reproducibility shown by the direct-acting positive control chemical, ethyl methanesulfonate (EMS), and also by methylnitrosourea (MNU) was very good. The data for EMS in liver and stomach in Phase 3 are shown in Figs 28 and 30.

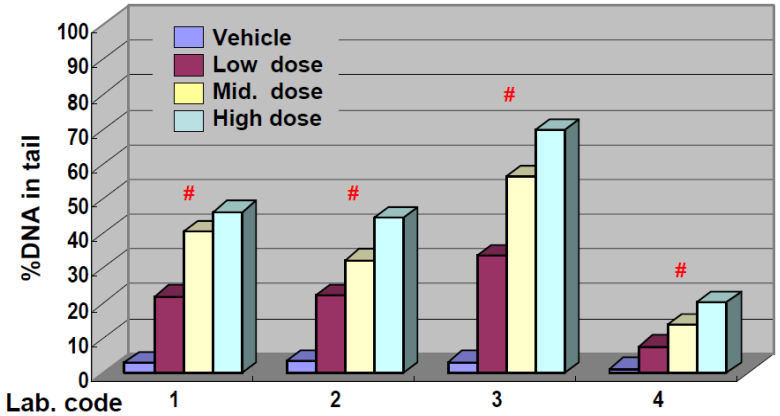


Fig. 28 Mean of %DNA in tail in Liver: EMS

 # Linear trend (P<0.05; two-sided)
Test condition
- ✓ **Animal: CD(SD) rat, male, 5 animals/group**
- ✓ **Dose: 0, 100, 200, 300 mg/kg, p.o.**
- ✓ **Administration: Twice (21 hours interval)**
- ✓ **Cell preparation: 3 hours after 2nd administration**

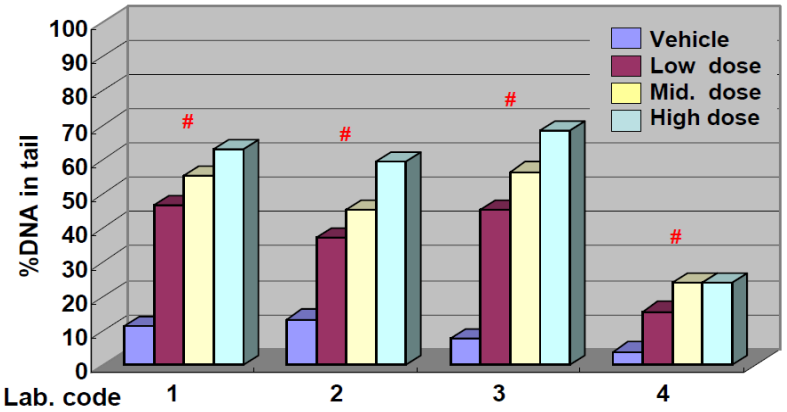


Fig. 30 Mean of %DNA in tail in Stomach: EMS

 # Linear trend (P<0.05; two-sided)
Test condition
- ✓ **Animal: CD(SD) rat, male, 5 animals/group**
- ✓ **Dose: 0, 100, 200, 300 mg/kg, p.o.**
- ✓ **Administration: Twice (21 hours interval)**
- ✓ **Cell preparation: 3 hours after 2nd administration**

The magnitude of the responses to EMS was lower in Lab.4 compared to the other laboratories. This was considered to be due to the shorter electrophoresis duration of 15 minutes used in Lab.4 (cf. Lab.1: 20 minutes in both organs, Lab.2: 30 minutes in both organs, and Lab.3: 20 minutes in the liver and 30 minutes in the stomach).

When dose responses for EMS at 2 dose levels were examined in Phase 3 (see Figs 1 and 2 below), Although the slopes of the responses (i.e. from 100 to 200 mg/kg of EMS) were similar for most laboratories, Lab 2 consistently showed a large increase in effect at 200 compared to 100 mg/kg in both liver and stomach than the other laboratories. The reasons for this are not known. However, the VMT considered that, since the protocol had not been optimized for validation purposes at this stage, the reproducibility of results between the laboratories was acceptable.

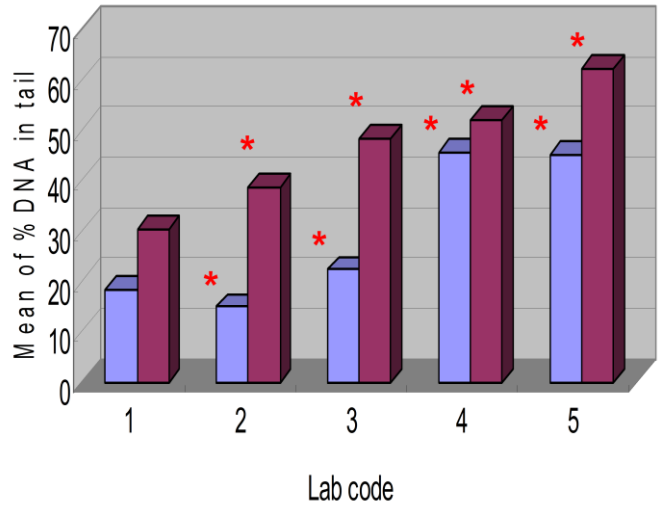


Fig 1. EMS liver, 100 & 200 mg/kg/day – Phase 3

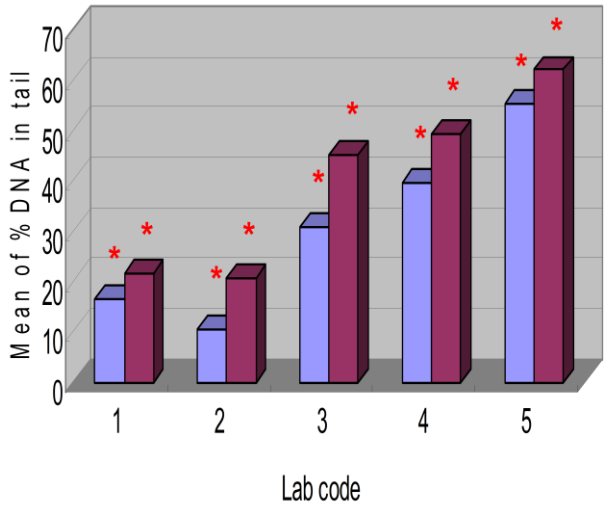


Fig 2. EMS Stomach, 100 & 200 mg/kg/day – Phase 3

The data for MNU in liver and stomach in Phase 3 are shown in Figs 32 and 34.

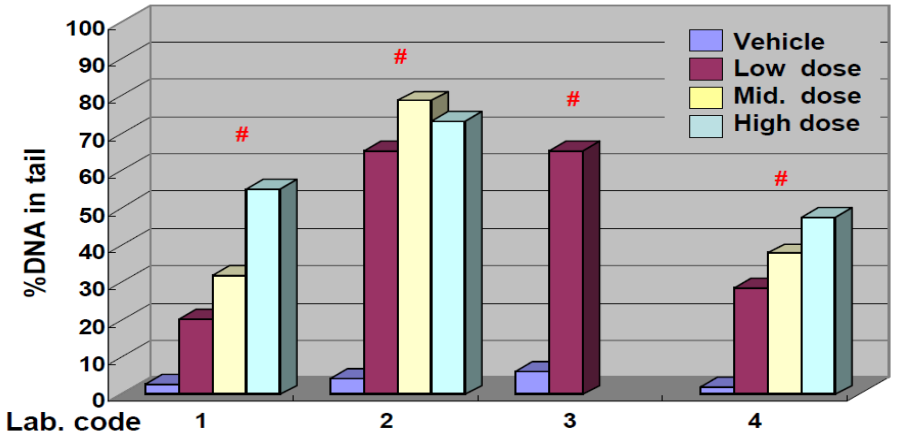


Fig. 32 Mean of %DNA in tail in Liver: MNU

 # Linear trend (P<0.05; two-sided)
Test condition
- ✓ **Animal: CD(SD) rat, male, 5 animals/group**
- ✓ **Dose: 0, 25, 50, 100 mg/kg, p.o.**
- ✓ **Administration: Twice (21 hours interval)**
- ✓ **Cell preparation: 3 hours after 2nd administration**

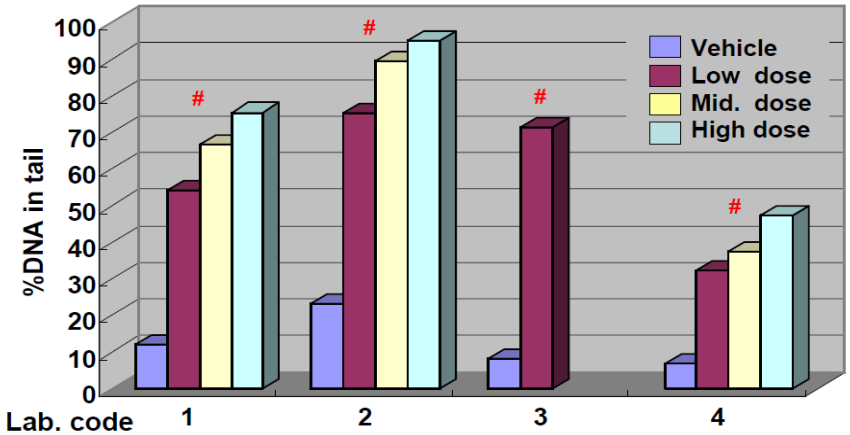


Fig. 34 Mean of %DNA in tail in Stomach: MNU

 # Linear trend (P<0.05; two-sided)
Test condition
- ✓ **Animal: CD(SD) rat, male, 5 animals/group**
- ✓ **Dose: 0, 25, 50, 100 mg/kg, p.o.**
- ✓ **Administration: Twice (21 hours interval)**
- ✓ **Cell preparation: 3 hours after 2nd administration**

Severe cytotoxicity was seen with MNU in Lab 3 at the top 2 doses. The reasons are not clear. However, a clear positive response was seen at 25 mg/kg/day which was similar in magnitude to that seen in Labs 1 & 2. The magnitude of responses to MNU was lower in Lab.4 compared to the other laboratories. This was again considered to be due to the shorter electrophoresis duration of 15 minutes used in Lab.4 (cf. Lab.1: 20 minutes in both organs, Lab.2: 30 minutes in both organs, and Lab.3: 20 minutes in the liver and 30 minutes in the stomach).

The data for the negative control chemical mannitol (MA) in liver and stomach in Phase 3 are shown in Figs 36 and 38.

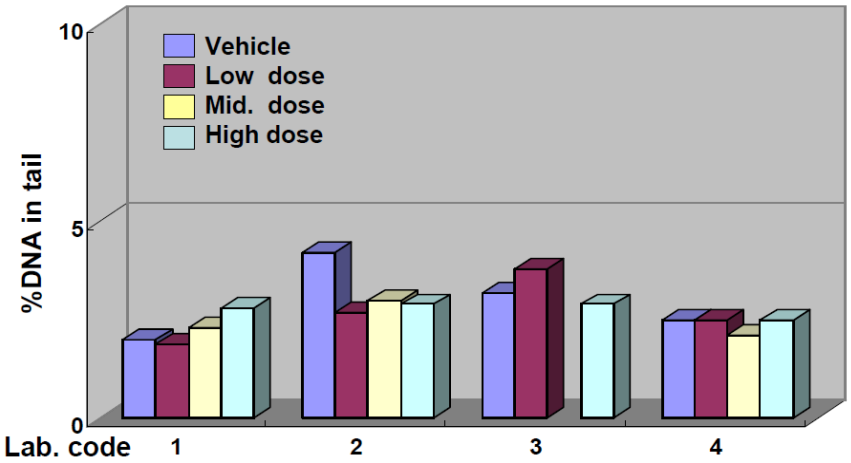


Fig. 36 Mean of %DNA in tail in Liver: MA

No statistical significance with Linear trend (P<0.05; two-sided)

Test condition
- ✓ Animal: CD(SD) rat, male, 5 animals/group
- ✓ Dose: 0, 500, 1000, 2000 mg/kg, p.o.
- ✓ Administration: Twice (21 hours interval)
- ✓ Cell preparation: 3 hours after 2nd administration

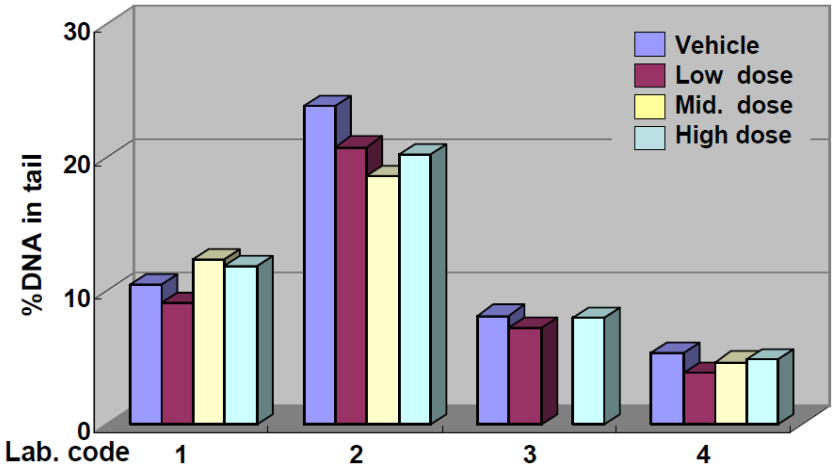


Fig. 38 Mean of %DNA in tail in Stomach: MA

No statistical significance with Dunnett (P<0.05; both sides) and Linear trend (P<0.05)

Test condition
- ✓ Animal: CD(SD) rat, male, 5 animals/group
- ✓ Dose: 0, 500, 1000, 2000 mg/kg, p.o.
- ✓ Administration: Twice (21 hours interval)
- ✓ Cell preparation: 3 hours after 2nd administration

The magnitude of the responses to MA seemed slightly higher in Lab.2 compared to the other laboratories, but the reasons are not clear. However, the vehicle control responses were also higher in this lab and MA gave a negative result.

The data for EMS in liver and stomach when used as a positive control in Phase 4-1 of the trial are shown in Figs 3 and 4 below.

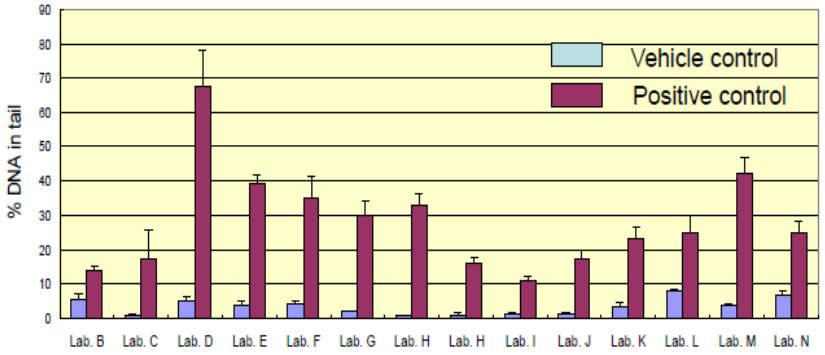


Fig 3. EMS (200 mg/kg/day as positive control) in liver – Phase 4-1

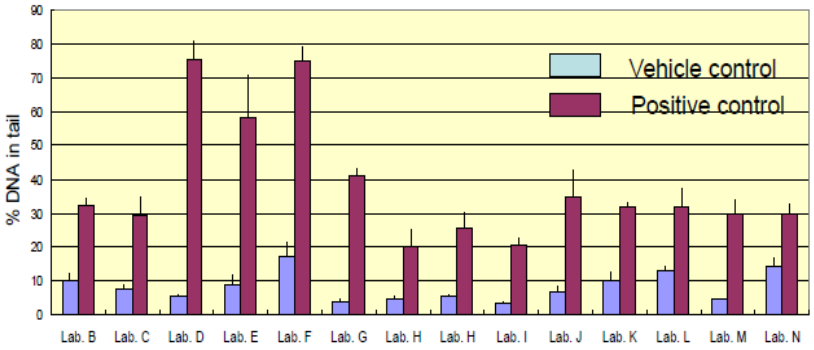


Fig 4. EMS (200 mg/kg/day as positive control) in stomach – Phase 4-1

The dose responses for EMS in liver and stomach as a coded compound in Phase 4-1 of the trial are shown in Figs 5 and 6 below.

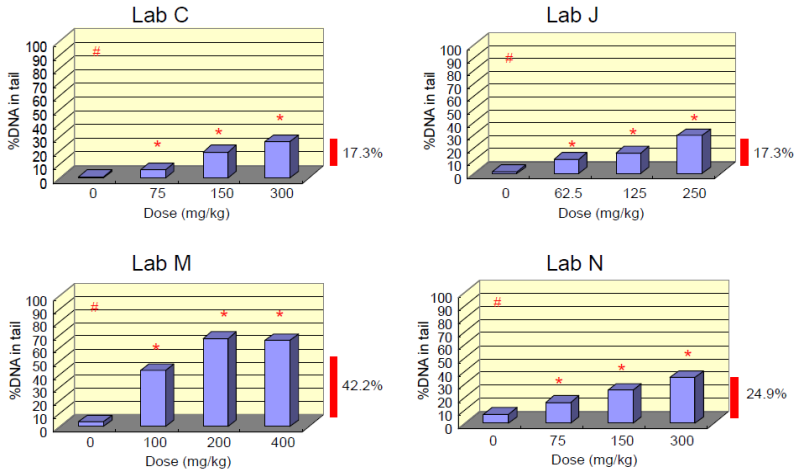


Fig 5. EMS dose-responses in liver as coded compound – Phase 4-1

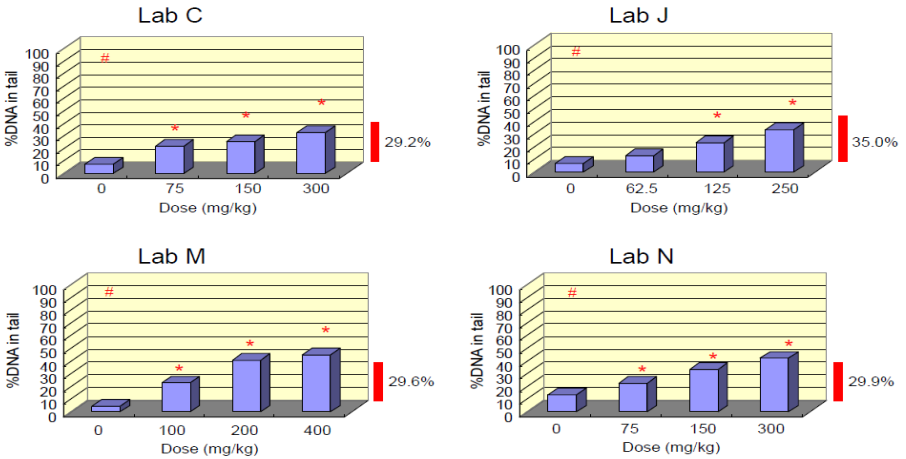
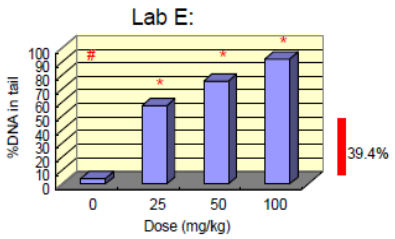
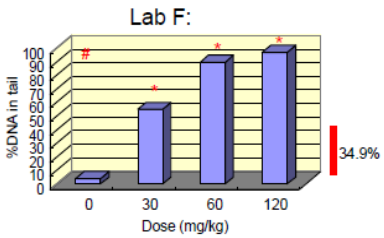


Fig 6. EMS dose-responses in stomach as coded compound – Phase 4-1

The dose responses for MNU in liver and stomach as a coded compound in Phase 4-1 of the trial are shown in Figs 7 and 8 below.





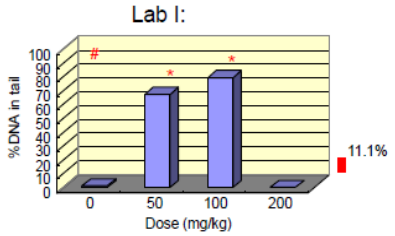
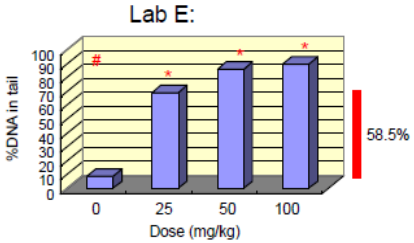
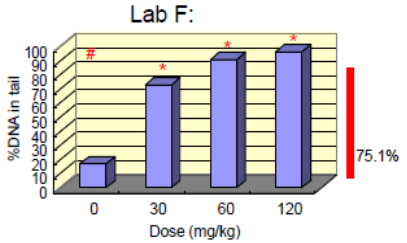


Fig 7. MNU dose responses in liver as coded compound – Phase 4-1





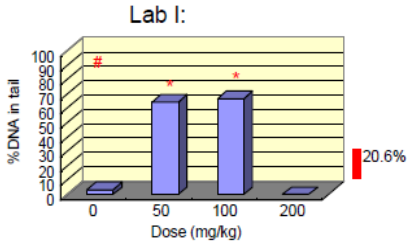


Fig 8. MNU dose responses in stomach as coded compound – Phase 4-1

The data showing the negative control chemical mannitol (MA) was negative in liver and stomach when tested as a coded compound in Phase 4-1 of the trial are shown in Figs 9 and 10 below.

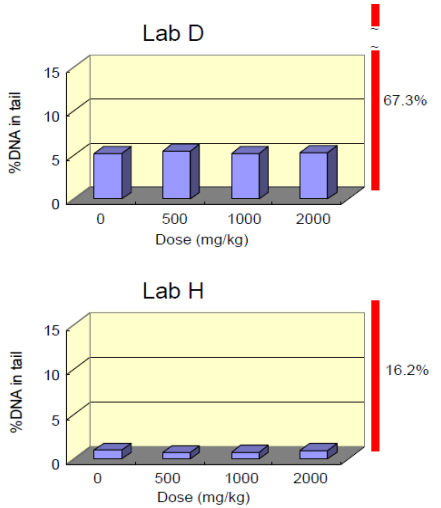


Fig 9. Mannitol as a coded compound in liver – Phase 4-1

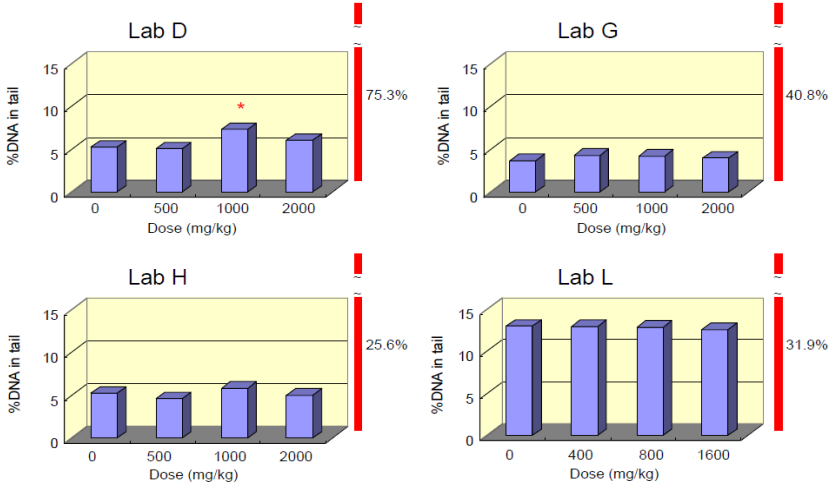


Fig 10. Mannitol as a coded compound in stomach – Phase 4-1

Thus, EMS and MNU not only showed good reproducibility of positive responses from a qualitative point of view, there was also generally good quantitative reproducibility across different experiments in different phases of the trial, and across different laboratories. Although MA showed a slight but statistically significant increase in Dunnett's test for the stomach at the mid dose level (1000 mg/kg/day) in Lab D, both Lab D and the VMT judged the response to be biologically insignificant because it showed no dose dependency. The reproducibility of qualitatively negative results with a known non-carcinogen (MA) was therefore also demonstrated. These results were quite consistent with those obtained for the same chemicals in the 3rd phase pre-validation study, confirming the robust reproducibility of the assay both over time and between laboratories.

However, the reproducibility shown by 2-acetylaminofluorene (2-AAF), the positive control chemical requiring metabolism, was poor. The data for 2-AAF as a coded compound in Phase 4-1 of the trial are shown in Figs 11 and 12 below.

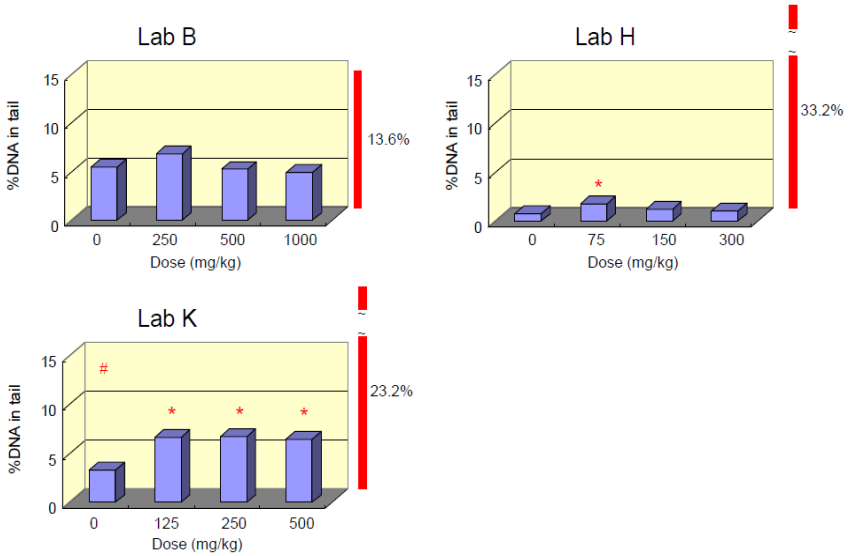


Fig 11. 2-AAF as a coded compound in liver – Phase 4-1

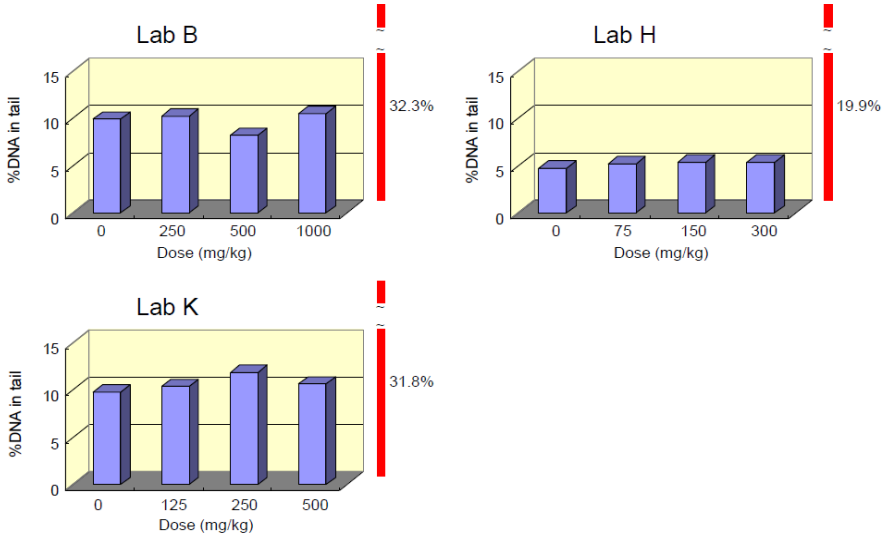


Fig 12. 2-AAF as a coded compound in stomach – Phase 4-1

Negative results in both organs were obtained in Lab B, but Lab B suggested that the higher dose levels up to 2000 mg/kg should be examined because there were no toxic signs in animals up to 1000 mg/kg/day. Lab H examined 2-AAF only up to 300 mg/kg/day based on information in the published literature, and judged it to be negative in both organs. Based on the lack of toxic signs at this dose, and data from the other laboratories, higher doses should have been used. In contrast, statistically significant increases in % DNA in tail were noted in the liver at all dose levels in Lab K, even though the highest dose level was only 500 mg/kg/day, and a negative result was obtained in the stomach.

Since the dose levels of 2-AAF used in the three laboratories participating in Phase 4-1 were not similar, the VMT considered that it would be difficult to assess the consistency of assay results in those three laboratories that tested 2-AAF. Finally, the VMT considered that 2-AAF was judged as inconclusive due to the variable testing conditions. Therefore the VMT decided that 2-AAF would be re-examined in Phase 4-2 of the validation study under the coded test chemical conditions. In the one laboratory that tested 2-AAF in Phase 4-2 of the trial, negative results were obtained both in liver and stomach. Other chemicals that require metabolic activation gave expected positive results (e.g. 2,4-diaminotoluene, *N*-nitrosodimethylamine), however, each was only tested

in a single laboratory and therefore intra- and inter-laboratory reproducibility could not be established. There may be some concerns that the *in vivo* Comet assay does not give reproducible responses with chemicals that require metabolic activation.

It was therefore decided to try to obtain additional data for the *in vivo* Comet assay to assess the intra- and inter-laboratory reproducibility of chemicals requiring metabolic activation. Two approaches were taken:

- Industrial laboratories were asked to provide any in-house data on testing of positive control chemicals known to require metabolic activation
- Data from the published literature (in particular the publications of Rothfuss *et al*, 2010 and Bowen *et al*, 2011 – papers appended) were reviewed.

Data from industrial laboratories

Letters were sent to a number of laboratories requesting data, and replies with data were received from seven different companies. These data are summarised in Appendix 1. It can be seen that:

- DMN was positive in the liver in 1 laboratory, but since it was also positive as a coded chemical at a similar dose in Phase 4-2 of the JaCVAM trial, it can be considered that acceptable inter-laboratory reproducibility has been achieved.
- Cyclophosphamide (CP) was positive in the liver in 1 laboratory, but was negative in another and showed inconsistent results in the liver in a third laboratory. Given the evidence that CP is effectively detoxified by GSH conjugation, it might be expected that it would not consistently produce DNA damage in the liver. Comets might only be detected when detoxification by GSH has been saturated.
- Acrylamide was positive in the liver in 1 laboratory. Since it was positive according to statistical analysis in Phase 4-2 of the JaCVAM trial, it can be considered that acceptable inter-laboratory reproducibility has been demonstrated.
- DMBA was positive in the liver in 1 laboratory when sufficiently high doses were tested. However, as it appears it was only tested once, and was not included in the JaCVAM trial, intra- and inter-laboratory reproducibility cannot be concluded from this.
- Aflatoxin B1 was tested in 1 laboratory and gave a positive response, but this was associated with histopathological changes and so the result was inconclusive. However, as it appears it was only tested once, and was not included in the JaCVAM trial, intra- and inter-laboratory reproducibility cannot be concluded from this.

- N-butyl-N-(4-hydroxybutyl)-nitrosamine was tested in 1 laboratory and gave positive responses at the same dose levels in both liver and urinary bladder in males and females of 3 different strains of rat. Therefore, intra-laboratory reproducibility was clearly demonstrated with this chemical.

The additional data supplied by the 7 laboratories on compounds not requiring metabolism (i.e. EMS, MMS, MNU and AZT) that were also included in the pre-validation and validation phases of the JaCVAM trial, further demonstrate the inter-laboratory reproducibility of the assay.

Data from Rothfuss *et al* (2010)

The following compounds expected to require metabolic activation were evaluated in the studies presented in this paper:

- benzo[a]pyrene (B[a]P) – not carcinogenic in liver, but induces DNA adducts and transgenic mutations in rodent liver
- 1,2- dimethylhydrazine (1,2-DMH) – liver carcinogenicity not tested, but induces DNA adducts, comets and UDS in rodent liver
- 2,6-dinitrotoluene (2,6-DNT) – liver carcinogen and induces DNA adducts and UDS in rodent liver
- dimethylnitrosamine (DMN) – liver carcinogen and induces DNA adducts, comets, UDS and transgenic mutations in rodent liver
- 2-amino-3-methylimidazo[4,5-f]quinoline (IQ) – liver carcinogens and induces comets and transgenic mutations in rodent liver
- 2,4-diaminotoluene (2,4-DAT) – liver carcinogen and induces UDS and transgenic mutations in rodent liver
- acrylamide (ACR) – not a liver carcinogen but does induce DNA adducts and comets in rodent liver, although it is negative for UDS and transgenic mutations.

In addition 1,2-dibromomethane (1,2-DBE), which is a liver carcinogen in females only, and induces comets and UDS in liver after ip administration, but does induce DNA adducts in liver, was included. Also, 2,6-diaminotoluene (2,6-DAT), which is a non-carcinogenic analogue of 2,4-DAT, and three non-genotoxic liver carcinogens (methapyrilene (MP), clofibrate (CFB) and phenobarbital (PHE)) were included.

Male rats received oral administrations of the test compounds, daily for two or four weeks. The top dose was meant to be the highest dose producing clinical signs or histopathological effects without causing mortality, i.e. the 28-day maximum tolerated dose. The liver Comet assay was performed according to published recommendations and following the protocol for the ongoing JaCVAM validation trial. Laboratories provided liver Comet assay data obtained at the end of the long-term (2- or 4-week) studies together with an evaluation of liver histology. Although each chemical was tested in a single laboratory (except for IQ which was tested in 2 laboratories), most of the test compounds were also investigated in the liver Comet assay after short-term (1–3 daily) administration to compare the sensitivity of the two study designs.

The key results can be summarised as follows:

- 1,2-DMH was positive in liver in both acute and repeat-dose protocols. It was also positive in liver in Phase 4-2 of the JaCVAM trial.
- 2,6-DNT was positive in liver in both acute and repeat-dose protocols.
- DMN was positive in liver in the repeat-dose protocol. It was not tested in the acute protocol. It was also positive in liver in Phase 4-2 of the JaCVAM trial.
- 1,2-DBE was positive in liver in both acute and repeat-dose protocols. It was also positive in liver in Phase 4-2 of the JaCVAM trial.
- IQ was positive in liver in the repeat-dose protocol and positive in 1 of 2 laboratories in the acute protocol.
- ACR was positive in liver in both acute and repeat-dose protocols. It was also positive in liver (according to statistical analysis) in Phase 4-2 of the JaCVAM trial.

B[a]P did not induce liver comets in either the acute or repeat-dose protocol. However, it is not a liver carcinogen. 2,4-DAT also did not induce liver comets in the repeat-dose protocol but was not tested with the acute protocol, which did produce a positive response in the JaCVAM trial. On the other hand, 2,6-DAT, which might have been expected to give negative results, was positive in liver in both acute and repeat-dose protocols. However, this is consistent with published findings (see Rothfuss *et al*, 2010 for references) and results in the JaCVAM trial.

These data therefore demonstrate acceptable intra-laboratory reproducibility (between acute and repeat-dose protocols in the same facility), demonstrate acceptable inter-laboratory reproducibility (in comparison with published findings and the JaCVAM trial), and demonstrate the applicability of the assay for detecting liver genotoxins that require metabolic activation.

Of the 3 non-genotoxic liver carcinogens, all were negative for comets in liver using the repeat-dose protocol. However, PHE induced liver comets with the acute protocol, as has been described in the published literature (see Rothfuss *et al*, 2010 for references). These data also demonstrate the applicability of the assay in giving negative results with chemicals expected to be non-genotoxic in liver.

Data from Bowen *et al* (2011)

The following compounds, expected to require metabolic activation were evaluated in the studies presented in this paper:

- 2-acetylaminofluorene (2-AAF) – administered ip
- benzo[a]pyrene (B[a]P) – administered ip
- cyclophosphamide (CPA) – administered orally
- dimethylnitrosamine (DMN) – administered orally

Male rats were dosed at 0, 24 and 45 h, and liver, whole blood and stomach were sampled for comet analysis three hours after the last dose. The Comet assay was performed according to published recommendations. In addition, bone marrow and peripheral blood were sampled at the same time for micronucleus evaluation.

The key results can be summarised as follows:

- 2-AAF induced comets in liver but not in stomach or blood when administered ip. It had given negative comet responses in all tissues when administered orally in initial trials. These effects are consistent with those seen in the JaCVAM trial.
- B[a]P induced comets in stomach but not in liver or blood. It had given negative results in all tissues when administered orally in initial trials, which is consistent with results obtained in the JaCVAM trial, and by Rothfuss *et al* (2010).
- CPA induced comets in stomach and blood but not in liver. The negative results in the liver were not clearly explained, but may be due either to efficient detoxification of reactive metabolites or to possible DNA crosslinking mediated by the metabolite phosphoramide mustard. The induction of comets in stomach may have been mediated via another pathway, or acid hydrolysis (due to the environment of the stomach) may have yielded a non-crosslinking mutagen
- DMN induced comets in liver and blood but not in stomach. These results are consistent with those obtained in the JaCVAM trial and by rothfuss *et al* (2010).

Thus, overall, there is good reproducibility for the detection of comets in liver and stomach by compounds requiring metabolism both within and between laboratories.

Conclusions

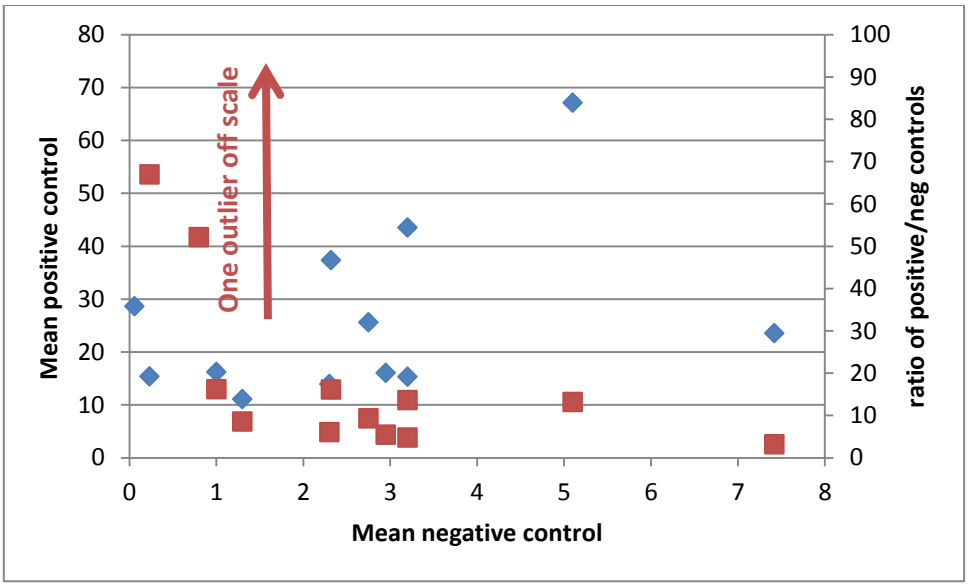
- From the JaCVAM trial, in-house and published results there is a reasonable amount of data supporting good qualitative reproducibility of responses for direct-acting chemicals, and those requiring metabolism, both within and between laboratories.
- Some quantitative variability was seen both within and between labs, but this has been seen in numerous collaborative trials across the decades and is not a surprise.
- Given the efforts already undertaken, it is unlikely that large amounts of additional useful data exist either in the published literature or in company archives.

Appendix 1: Data from 7 laboratories

Laboratory	Compounds tested	Study design	Results	Overall call	Comments
BMS	CP (10mg/kg/d)[a] ENU (10mg/kg/d)[b] MNU (2.5mg/kg/d)[c]	1-month Oral Integrated Pig-a, MN, and Comet assay. Male Sprague-Dawley rats. 150 Nuclei analysed per sample, Comet IV image analysis. Rats were dosed with all 3 positive controls by either PO or ip route on the days indicated; (a)Days 1-2, 27-28 with CP by PO route; (b)Days 1-3 with ENU by ip route; (c)Day 30, 3 hr before necropsy with MNU by PO route.	Significant increase in DNA damage in liver cells	Positive	Brain (EMS) and blood (MNU) data also available (po)
Merck	EMS 200 mg/kg (PO) [1X and 2X]	Followed JaCVAM protocol. 1X = rats dosed once, 3hrs prior to necropsy; 2X = rats dosed twice 23 and 3hrs prior to necropsy	Significant increase in DNA damage in liver cells	Positive	Results provided for different vehicles. Here presented overall results. Stomach data also available (positive, increase in DNA damage)
	DMN 2.5mg/kg (PO)	Followed JaCVAM protocol.	Significant increase in DNA damage in liver cells	Positive	
	Acrylamide (PO) 50 mg/kg	Followed JaCVAM protocol.	Significant increase in DNA damage in liver cells	Positive	
ILS (NTP data)	Cyclophosphamide	MN/Comet studies (4 days of dosing) for the NTP that either tested CP, or utilized CP as a positive control. Followed JaCVAM protocol but there were some differences in electrophoresis temperature, however it does not change the overall case based on concurrent controls	CP didn't reliably test positive in the liver, but it did in other organs (leukocytes and duodenum). Data published, Recio et al 2010 J. Toxicol. Sci Vol35, No.2, 149-162. Raw data was provided.	Equivocal	One of the debates on CP is cross links vs. GSH detoxication for lack of response for CP. ILS showed that it is effectively detoxified by GSH conjugation.
BioReliance	DMBA 2.5, 5, 10 mg/kg	29 days oral administration. Integrated Pig-a, MN, and Comet assay. Samples collected 3-4 hours after last dose. Followed JaCVAM protocol.	No increase in DNA damage in liver cells	Negative	Study repeated at higher doses
	DMBA 10, 50, 100 and 200 mg/kg	Acute dosing. Samples collected 3-4 hours after last dose. Followed JaCVAM protocol.	Increased DNA damage in liver cells	Positive	

J&J	CP	Single oral dose oral; liver of male SD rats; tissue sampling at 3 and 24 hour post-dose; Saline was used as vehicle control; No histopathology was conducted; Top dose of 30 mg/kg was not the MTD, but was based on published data (Ashby and Beije, MR 150 (1985): 383-392) demonstrating that dose levels of > 30 mg/kg were toxic to the liver. Interesting to note is that the UDS with CP was negative in this publication.	No increase in DNA damage in liver cells	Negative	
	Aflatoxin B1	3-day repeat dose oral comet assay in liver and blood of male SD rats following the JaCVAM protocol Top dose of 1 mg/kg/day was considered the MTD	Increase in DNA damage in the liver is associated with histopathological findings	Inconclusive	
Roche	EMS	JaCVAM Protocol, with the exception that PI instead of SYBR Gold was used for DNA staining	Pos (Liver, stomach)	Data from the same animals are available to compare with Novartis	
	MMS		Pos (Liver, jejunum, bladder - only 2 dose levels); blood (day 22)	High dose not the MTD for an acute treatment but 28 d treatment	
	Temozolomid		Pos (Liver, jejunum, blood)		
	AZT		Pos(Liver, stomach, blood)	Treatment over 7d	
Japanese lab (not named)	N-butyl-N-(4-hydroxybutyl)-nitrosamine	JaCVAM final protocol with 3 different strains of rat	Positive (liver and urinary bladder) at similar doses in all strains, male and female	Positive	

Annex 2: Analysis of critical variables in the comet assay



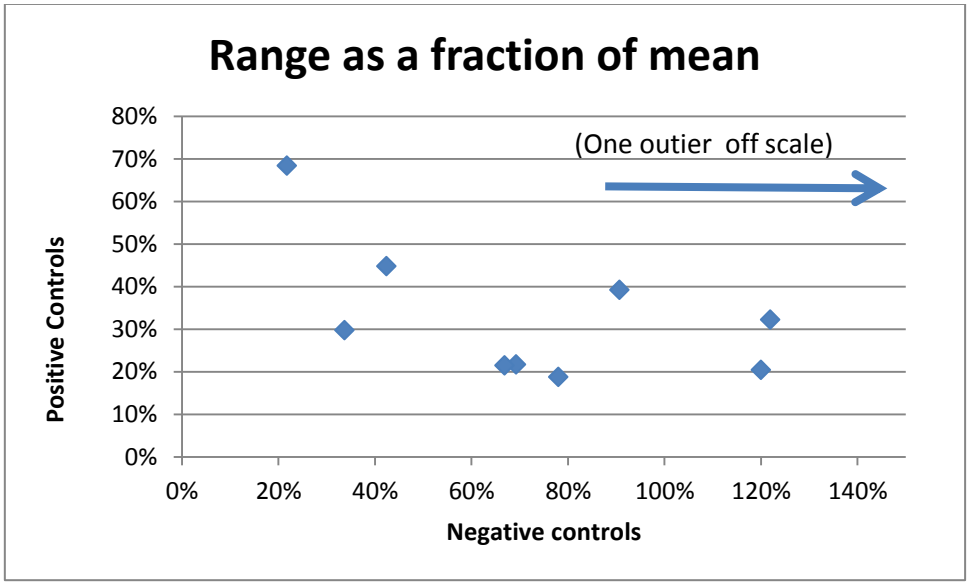


Table 1: Analysis of Critical Variables in the Comet Assay for 4 Laboratories that Showed Extreme (high and low) Responses with EMS in the JaCVAM Trial

Critical Variable	Lab 1 (Lab I)	Lab 2 (Lab H)	Lab 3 (LAB M)	Lab 4 (LAB D)
Dosing Regimen				
Vehicle	Physiological saline	0.9% Physiological saline	Sterile Saline	Purified water
Positive Control Id	EMS	EMS	EMS	EMS
Positive Control vehicle	Physiological saline	0.9% Physiological saline	Purified water	Physiological saline (20 mg/ml)
Positive control source	Sigma-Aldrich	Sigma-Aldrich	Sigma-Aldrich	Sigma-Aldrich
Positive Control Dose	200 mg/kg/bw	200 mg/kg/bw	200 mg/kg bw	200 mg/kg bw
Postivie control Dosing Frequency	twice	twice	twice	twice
Animals				
rat/mouse/other	Rat	Rat	Rat	Rat
strain	CRL:CD(SD), SPF	Sprague Dawley CD1 rats	Sprague Dawley	Sprague Dawley
male/female	Male	Male	Male	Male
Tissue preperation				
Tissue	Liver	Liver	Liver	Liver
Time tissue kept after dissection (prior to slide preparation)	Suspensions prepeared within 22 min of sacrifice.	Immediately?	Maximum 1 hour	Not reported
Temperature of tissue kept after dissection	Cold	Kept on ice	Kept on ice	Kept on ice
Preparation method of single cell suspension	In the liver, portions of the liver (about 5-mm square from the left lateral lobe) were minced with a pair of scissors. The cells were suspended in 5 mL of the cold homogenizing buffer, and the cell suspension was strained through a cell strainer.	The liver was placed in 1 mL ice cold buffer and ruptured with tweezers about 8 to 10 times. The cells were resuspended using a 1 mL pipette.	cut into small pieces in mincing solution. The pieces of liver were then pushed through bolting cloth (pore size of 150 µm) with approximately 4 mL of mincing solution to produce single cell suspensions.	Not described in detail.
Agarose concentration (mix with cells)	Mixed 1:9 with 0.5% low-melting agarose and 0.15 ml placed in a well on a MAS coated glass slide within 44 min of single cell preparation.	30 uL aliquot of liver cell suspension was mixed with 225 uL 0.5% low melting agarose (LMA) and 30 uL added to each slide and covered with a cover slip.	An aliquot of each single cell suspension was added to 0.7% low melting point agarose (LMA) held at approximately 37 ± 1°C. 100 uL of cell suspension/agarose mix was subsequently placed on to a slide that had been previously coated in normal melting point agarose (NMA)	Sample added to an appropriate amount of 0.5% low-melting agarose
Test Variables				
Lysis time/temperature	overnight, refrigerator temp. Started within 5 min. of slide preparation	overnight, refrigerator temp.	overnight, 1-10 °C	overnight, refrigerator temp.
Unwinding buffer pH	Not reported	Not reported	pH>13	Not reported
Unwinding buffer temperature	3.5-4.5 °C	4 °C	Not reported, but probably 1-10 °C	2-8 °C
Unwiding time	20 min	30 min	30 min	20 min
Electrophoeris buffer pH	Not reported	Not reported	Not reported	Not reported
Electrophoresis buffer temperature	3.5-4.5 °C	4 °C	Not reported, but probably 1-10 °C	Not reported, but probably 2-8 °C
Electrophoresis time	20 min	20 min	40 min	30 min
Volts/cm	0.7 v/cm (26 V) at 299-324 mAmp.	0.7 V/cm, 300 mAmp	0.7 V/Cm & Constant 300 mAmp	18 V (0.7-1.0 V/cm) approx 300 mAmp
Staining				
Dried slides or hydrated slides	Dried	Hydrated	Dried	Not clear
Dye	SYBR Gold	Propidium iodide	Ethidium Bromide.	SYBR Gold

Image analysis system				
Analysis software	Comet Assay IV, Perceptive	Comet Assay IV, Perceptive	Comet Assay IV, Perceptive	Comet Assay IV, Perceptive
Results				
Vehicle Control %Tail DNA (mean group value)	1.3	1	0.8	5.1
Vehicle Control Group Range (min-max)	0.9-1.8	0.2-1.4	0.52-1.24	4.53-6.69
Vehicle: Range as a fraction of mean	69.2%	120.0%	298.1%	42.4%
Postivie Control %Tail DNA (mean group value)	11.1	16.2	41.7	67.1
Postivie Control Group Range (min-max)	9.9-12.3	13.9-17.2	35.05-55.57	53.19-82.7
Positive: range as a fraction of mean	21.6%	20.4%	60.0%	44.7%
ratio of positive/neg controls	8.5	16.2	52.1	13.2
Are the mean presented here: the means of individual animal means, or means of individual animal medians	Mean values of individual animals	Not reported	Median values of individual animals	Mean values of individual animals
Historical positive control		Not reported	min/max 15/69: 4.5 fold, 95% 2.8 fold N=53	min/max=0.29/8.21 4.9 fold Mean 3.4 N 111
Historical negative control	0.55/1.77 (3 fold)	Not reported	min/max= 0.13/14.7: 113 fold, 95%: 27 fold N=59	Min/Max= 12/59 28 fold mean 42 N=100
electrophoresis box	Not reported	Not reported	Not reported	
how much electrophoresis buffer	slides immersed	Not reported	Not reported	
slides: commercial? In house?	commercial: MAS coated.	Not reported	seems to be in house	in house by dipping in 1% agarose.
fresh lysis buffer?	Triton X and DMSO added when used.	Not reported	Not reported	
Fresh electrophoresis buffer?		Not reported	Not reported	
		DMSO added to mincing buffer just before necropsy.		
		not clear if constant voltage or current		

Table 2: Analysis of Critical Variables in the Comet Assay - Other Laboratories that Participated in the 1st Step of 4th Phase Validation Study

Critical Variable	Novartis	Merk	Mitsubishi	IET	Janssen	Health Canada	FDSC	Bayer	ILS	Bioreliance
Dosing Regimen										
Vehicle										
Positive Control Id										
Positive Control vehicle										
Positive control source										
Positive Control Dose										
Postivie control Dosing Frequency										
Animals										
rat/mouse/other										
strain										
male/female										
Tissue preperation										
Tissue										
Time tissue kept after dissection (prior to slide preparation)		slides made within 5 min of euthanasia		Approx. 1 hr	Within 1 hr	time from sacrifice to gel casting ~ 5 min	about 4 hours for dissection and slide preparation	not reported	Not reported	Not reported
Temperature of tissue kept after dissection		ice cold			on ice	use ice cold buffers	on ice	used cold buffer	on ice	in chilled mincing solution
Preparation method of single cell suspension		roughly minced	minced with a pair of fine scissors about 100 times to release the cells. The cells were gently moved about 15 times with a 3-mL cold mincing buffer by a pipette and suspended. Then, the cell suspension was strained through a cell strainer (pore size: 40 µm) and placed on ice.	Approx 3 hrs	A standardized portion of liver will be minsed with a fine pair of scissors to relaease the cells …[then] strained through a cell strainer to remove lumps…	- mince with fine scissors, add 10ml Mincing buffer - store suspension on ice for 15-30 sec to allow for large clumps to settle - use supernatant to prepare Comet slides. Time from sacrifice to gel casting ~ 5 min	minced with a pair .of fine curved scissors about 100 times. The minced cells were suspended in 2 mL of the cold mincing buffer by gentle pipetting about 5 times. Each cell suspension was passed through a Cell Strainer (pore size: 40 flm) to remove lumps and the passed suspension was placed .on ice until prepare Comet slides. Cells from the suspension from one negative control animal used to estimate $2x10^6$ cells/ml for all animals.	Tissue was disintegrated, transferred to a beaker with at least 60 ml mincing buffer and filtered through gauze (70).	A 2-3 mm2 section was placed in a cryotube containing 1 ml of cold mincing solution and rapidly (~30 sec) minced until finely dispersed	A portion of the left liver lobe, placed in cold mincing solution, was cut with fine scissors to release the cells.. The cell suspension [was] strained using a 40 ?m cell strainer and then immediately used in preparation of comet slides.
Agarose concentration (mix with cells)		30 ul of supernate (minced matieral allowed to settle briefly) added to 300 ul LMA.	Cell density of the prepared cell suspension was adjusted to about 2.0 × 105 cells/mL. Each cell sample (40 µL) was mixed with 0.5% low melting agarose gel (360 µL), and the mixture (40 µL) was placed onto each well of a slide	0.5% diluted to 0.45%	appprox 25 ul cell suspension added to 300 ul 0.5% LMA . 75 ul used per sample	~5 ul cell suspension in tube, then add 500 ul pre warmed 45 C 0.5% LMA. Use 75 ul per well.	40 ul of cell suspention was mixed with 360 ul 0.5 %LMA at 37 C. 150 ul of this mixture was layered on an agarose coated slide and covered with a cover glass	300 ul [liver cells] were pelleted at 116xg for 6 minutes. Resulting pellets were mixed with 75 ul LMA (0.7%) and applied onto slides (76 x 26 mm, Menzel, Germany) which were already precovered with a layer of 1 % normal melting agarose.	A portion of the cell suspension … was empirically diluted with a fresh aliquot of 0.5% [LMA] at 37-45°C, and layered onto conventional slides precoated with 1% normal melting agarose. The volume of the cell suspension did not decrease the percentage of low melting agarose by more than 10% (i.e., not below 0.45%).	From each liver cell suspension, an aliquot of 5 uL was mixed with LMA (0.5%). The cell/agarose suspension was applied to glass microscope slides, previously coated with normal melting agarose
Test Variables										

Lysis time/temperature		overnight 4-10 oC	Overnight (~18 hrs) in refregerator in dark	4 C for 2 days	at least overnight 2- 8 C. pH 10.0	overnight in refrigerator	overnight 2-6 C	overnight, < 8 C	overnight in refrigerator.	at least 24 hrs, 2-8 C
Unwinding buffer pH					>13	13.3	13	>13	>13	>13
Unwinding buffer temperature		6-8 °C	4 C			~ 6 C	5-6 C	<10 C	<10	2-8 C
Unwiding time		20 min	20 min		20 min	20 min	20 min	20 min	20 min	20 min
Electrophoeris buffer pH	Not reported	>13	>13 at preparation and 14.35 at use	>13	same as unwinding buffer	13.3	13	>13	>13	same buffer
Electrophoresis buffer temperature	6 °C	6-8 °C	4 C	4 C	4-8 C	4-6 C (recorded at start and end)	6-8 C	<10	<10	2-8 C
Electrophoresis time	40 min	20 min	20 min	20 min	30 min	20 min	20 min	20 min	20 min	30 min
Volts/cm	27 V , 300 mA	0.7 constant V/cm, approx 300 mAmp	0.7 V/cm constant voltage. 300-310 mAmp.	0.7 V/cm, 282-300 mAmp	0.7 V/cm approx 300 mAmp	17 V, 287-294 mAmp.	0.7 V/cm constant voltage. 300 mAmp	0.7 V/cm 300 mAmp	0.7 v/cm (25 V), 297-301 mAmp	7 V/cm
Staining										
Dried slides or hydrated slides	Not Clear	dehydrated with EtOH	dehydrated with EtOH	dehydrated with EtOH		not clear probably hydrated	dehydrated with EtOH	dehydrated with EtOH	dehydrated with EtOH	dehydrated with EtOH
Dye			SybrGold							
Image analysis system										
Analysis software										
Results										
Vehicle Control %Tail DNA (mean group value)	0.23	2.95	2.3	2.75	0.06		3.2	7.42	3.2	2.32
Vehicle Control Group Range (min-max)	± 0.05(S.D.)	1.96-3.90	2.03-2.53	±0.78(SD)	±0.04(SD)	data not present	1.2-5.1 (SD 6.8)	6.5-9.0 ± 0.8 (SD)	2.0-4.9 (SD 0.48)	1.31-2.86 (± 0.62 SD)
Vehicle: Range as a fraction of mean		78.0%	21.7%				121.9%	33.7%	90.6%	66.8%
Postivie Control %Tail DNA (mean group value)	15.4	16.03	13.9	25.6	28.6		43.5	23.56	15.3	37.33
Postivie Control Group Range (min-max)	3± (S.D.)	14.66-17.93	9.51-19.4	±6.3(SD)	±1.6(SD)	data not present	36.5-50.6 (SD11.2)	19-26, ± 2.3 (SD)	11.9-18.2, SD 1.13	33.3-40.5 (± 2.60 SD)
Positive: range as a fraction of mean		18.7%	68.3%				32.2%	29.7%	39.2%	21.4%
ratio of positive/neg controls	67.0	5.4	6.0	9.3	476.7		13.6	3.2	4.8	16.1
Are the mean presented here: the means of individual animal means, or means of individual animal medians	Mean of individual animals (Tail mommemt)	Mean of indivudiual animals	Means of individual animals	means of individual animals	means of individual animals	data not present	individual animal means. Medians also calculated.	calculated both mean of means and mean of medians	individual animal means	means of individual animal means
Historical positive control	Not reported	Not Reported	Not Reported	Not Reported	28.9 (20.6-40. 1.9 fold, SD 5.3)N=20	data not present	Not reported	Not reported	Not reported	20.7 (12.55, 5.5-80.3 14 FOLD)
Historical negative control	Not reported	Not Reported	Not Reported	Not Reported	1.94 (0.1-13., 130 fold SD 2.1) n=179	data not present	Not reported	Not reported	Not reported	2.4 (1.45, 0.31-8.42 27 fold)
electrophoresis box		SciPlast Comet40	Not Reported	Not Reported		Cleaver Scientific	Biocraft	Not reported	Not reported	Not reported
how much electrophoresis buffer		Slides immersed	enough to achieve .3-.31 Amp.	Not Reported		1.8 L	1 liter, amount adjusted to control current.	Not reported	Not reported	Not reported
slides: commercial? In house?	not described		Trevigen slides	Appears to be in house	Coated with agarose in house	Trevigen	prepared in house 2 days before use	appear to be prepared in house	appear to be prepared in house	appear to be prepared in house
fresh lysis buffer?	Not reported		Triton X and DMSO added on the day of use and refrigerated at least 30 min.	Not Reported	Triton X and DMSO added just before use.	Use within 1 week	DMSO and triton added just before use	DMSO and TritonX added on the day of use	DMSO and TritonX added fresh	Not reported
Fresh electrophoresis buffer?						NaOH only use within a day	prepared the day before			Not reported
			DMSO added to mincing buffer just before use		note that the neg control is very low w.r.t. the historical range.			mincing buffer prepared on day of procedure	DMSO added to Mincing buffer fresh.	