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

Five males for the validation study (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)	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.

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, which enable us to detect comet and micronucleus at the same time (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.

Test compound	0 hr	24 hr	45 hr	48 hr
or	|----------	----------|----------	----------|----------	----------|
Vehicle (negative control)	1st admin	2nd admin	3rd admin	Sacrifice

EMS (positive control)	0 hr	21 hr	24 hr
	|----------	----------|----------	----------|
	1st admin	2nd admin	Sacrifice

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~~ third administration of a test substance and at 3 hours after second treatment of EMS ~~treatment~~, consistent with Section C "Animal Welfare and 3Rs". The stomach and portions of 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. 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 6). ~~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 (see note 7)~~(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 during 20 minutes ~~(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~~ 300 mA (see note 8). 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 (see note 9) ~~(Notes: to be re-evaluated after statistical analysis)~~. Approximately 10 areas/slide should be observed at 5 cells or less/field (see note 10)~~(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 (see note 11)~~This instruction means that the automatic image analysis results will be basically accepted as they are. However the following cases will be excluded from the comet data analysis: the automatic recognition is judged incorrect (e.g. the center of nucleus is not recognized correctly); and the staining of nucleus and/or migration is judged wrong. Add pictures in an appendix, and VMT will prepare a color atlas to instruct how to judge comet and hedgehog soon 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 (see note 12), 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 13)~~Notes: at Atagawa meeting held on March 13-14, 2008, there were some discussions about necessity of tail length and Olive tail moment. Again, there was brief discussions about this point at Osaka meeting on February 4, 2009. 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~~ both sides test (P<0.05) and Trend test (P<0.05) ~~is~~ will be ~~also~~ applied to "Effect" ~~for data analysis~~ to judge positive or negative as assay results. For the positive control group, the Student's t-test (P<0.05) will be applied to the "Effect".

3. DATA AND REPORTING

3.1.1. Treatment of results

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

3.1.2. 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.

3.1.3. Study report

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

3.1.3.1. 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.

3.1.3.2. 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.

3.1.3.3. Reagents to prepare reagent solutions

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

3.1.3.4. 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.

3.1.3.5. 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.

3.1.3.6. 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

1) We will evaluate with data of the 2nd and the 3rd phase validation studies whether or not three (or four) animals are sufficient in the positive control group to show statistically significant increase in the Effect (diff. and ratio) with the student's t-test ($P<0.05$). If there is no difference in the statistical analysis results between five animals and fewer animals, we will use fewer animals as the positive control group. If changed, the VMT will inform participants of the modified animal number with an amendment of the study plan when the validation studies are ongoing. Regarding the other groups, we will decide the appropriate number of animals/group afterwards based upon power calculation.
2) We will likely need to specify shelf life for some solutions as we reconcile lab-specific protocols.
3) The VMT deeply discussed at Osaka meeting held on Feb. 4-6, 2009 how a preliminary dose-finding study should be done practically to chose an appropriate high dose level, because selection of a reasonable 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.
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 needed to administer EMS three times as well as the other test chemicals.
5) MN and Comet analysis for bone marrow and/or peripheral blood are just optional in this validation study.
6) The size of the liver portion will be at the discretion of the laboratory, because there is no suggestion that should be standardized until now thorough this validation effort.
7) 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.

8) Under those electrophoresis conditions, it will be expected to obtain an average DNA migration in the negative control group of 1-8% DNA in the tail for the liver, and 1-20% DNA in the tail 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 enable to detect decrease in % DNA in tail. The decrease in DNA migration is expected in cross-linkers, and it may be more effective that the negative control value keeps higher levels within the above range if such agents are intended to be detected using Comet assay. If the negative control average deviates from the range, the electrophoresis condition will be adjusted to come within the range.
9) To be re-evaluated after statistical analysis.
10) In order to obtain suitable areas for observation, dilution of cell suspension may be required during the single cell preparation process.
11) This instruction indicates if analyzable by software then 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. A few pictures are added in an appendix of this protocol, and the VMT will prepare a color atlas to instruct how to distinguish comet and hedgehog.
12) 'Tail length' is defined as 'Tail migration' in some image analyzers such as Comet IV.
13) At Atagawa meeting held on March 13-14, 2008, there was some discussion about necessity of tail length and Olive tail moment in this validation study. Again, there was brief discussion about this point at Osaka meeting on February 4, 2009. As a consensus in this validation study, these parameters are no longer necessary to analyze statistically, because % DNA in tail seems a sufficient endpoint for validation. But data on tail length and tail moment will continue to be collected in this validation study in case it is needed to analyze the data in future.

6. 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.

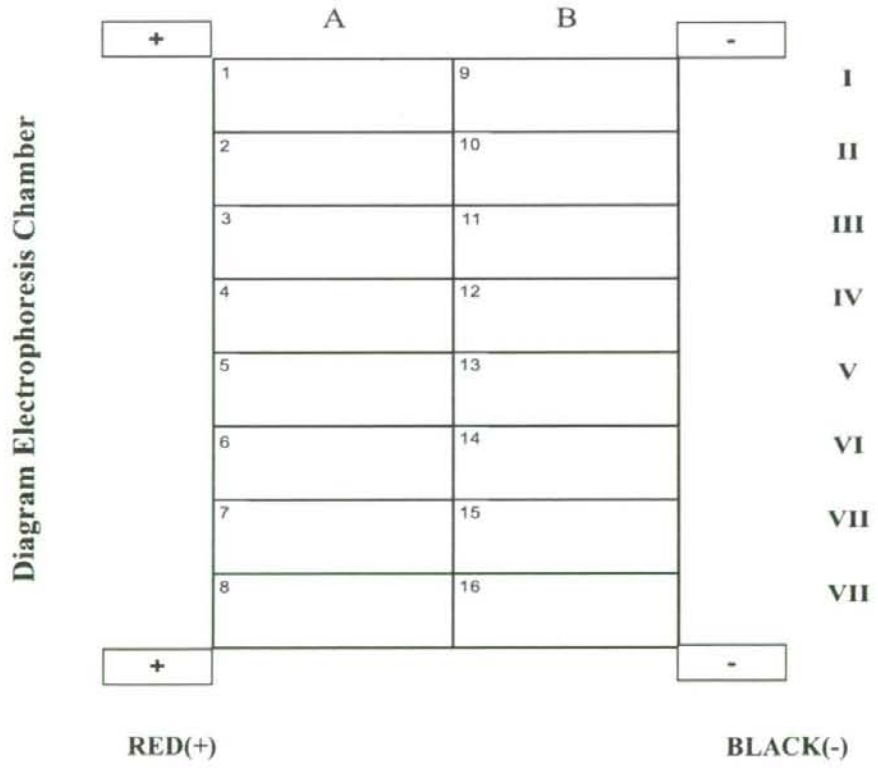
Olive PL, et al., Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cell using the "comet" assay. Radiat. Res., 122, 86-94, 1990.
Tice RR et al., Single cell gel/Comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ. Mol. Mutagen., 35, 206-221,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



Draft Minutes

The 5th meeting for

the International Comet assay validation study

Date: February 4, 2009, 9:00 a.m.-February 6, 3:00 p.m.

Venue: Sango and Suisho, 3F, Kansai Airport Washington Hotel, Osaka, Japan

February 4

Welcome address (Chair: Dr. Makoto Hayashi: Anpyo-Center)

1. The chair opened the meeting and welcomed participants. He introduced the topic and gave a presentation on the final goal of this project and the object of the 5th meeting for the International Comet assay validation study in Osaka, 2009 (Osaka meeting). He described the final goal of this project establishes the OECD Test Guidelines of comet assay and the object of the Osaka meeting is to understand the current situation of *in vitro* and *in vivo* comet assay.
2. The chair emphasized that participants ideally are present as members of the Validation Management Team (VMT) or the delegate of the participated laboratories. The Chair introduced Dr. Masamitsu Honma (NIHS) and Dr. Yoshifumi Uno (Mitsubishi Tanabe Pharma Corporation) as the acting co-chairs together with the chair, who were approved by the meeting.
3. The chair also announced that the standard protocol ver. 13 should be revised in accordance to the activity of ICH, EFPIA initiative collaborative study of comet assay incorporated into repeat-dose general toxicity study to fulfill the 3Rs of animal experiment. Namely, 3 treatments (0, 24, and 45 h) before sacrifice animals at 48 h after the first treatment instead of 2 treatments describes in the protocol ver. 13. This protocol can be used for the combination study of the micronucleus assay and the comet assay.
4. Participants introduced themselves to the meeting (for a list of participants see Annex 2).
5. Dr. Hajime Kojima (JaCVAM, NIHS) explained the general information in this meeting.

In *vitro* Comet-international validation study (Chair: Dr. Honma)

To understand the current situation of *in vitro* comet assay

1. To summarize Phase I

The mainly phase I data were presented by Dr. Honma.

Statistical consideration on phase I study by Mr. Masaya Suzuki (Anpyo-Center)

- 5 laboratories and 5 chemicals/laboratory were presented.
- 2AA: Variation may depend on different S9mix (only 2 +ve)
  - Hatano Research Institute (HRI) showed data that the different S9mix showed different results
- For the statistical analysis, one tail vs. two tails was discussed: no concrete conclusion.
- Flow: wording mean of relative cell survival …
- Log transformation for concentration was proposed by Dr. Lovell to show clearer dose-response relationship: Action, re-calculation and re-draw graphs by M. Suzuki.
- Failure based on protocol ???
- Generally, the variation in the comet assay can be smaller than Chromosome aberration assay.

2. To discuss on Phase II

The phase II study has not yet completed and not be able to break codes of chemicals. Thus, we could not discuss precisely based on the chemical information.

- 3 laboratories completed (dead line by the end of March)
- Presentation by the participating laboratories
- BR (BioReliance Corporation) had problem of KCl which for without S9 mix, then they used culture medium instead of KCl (They want to repeat experiments without S9 mix)
- Acceptance criteria: Hedgehog, >20%, the all concentration above that showed >20%
- Negative control. Solvent, KCl/S9 mix

By the end of May, all data should be collected

3. To discuss the future activities

Discussion and consensus

For Phase III studies, number of laboratories and number of chemicals were discussed. The plan will be proposed by the VMT.

February 4

Workshop - Image Analysis with Comet IV (Chair: Dr. Hayashi)

Dr. Hayashi expressed the importance of distinguishing between hedgehogs that is not genotoxic but related to cell-death, e.g., apoptosis and necrosis, and genotoxic comet strong positive cells. This may lead false-positive, which is the biggest concern of this assay. The group basically agreed the importance of the identification of the non-genotoxic hedgehog and understood this workshop. The main purpose of the workshop is to make consensus of the definition of hedgehog

- Comet and Hedgehog, which is which? : Dr. Madoka Nakajima and Dr. Jin Tanaka (Anpyo-Center) prepared the example images and all participants voted their judgment by their experience and intuition.
- After the vote to about 100 images, all questionable images were discussed one by one and discussed and agreed. All agreed the importance of definition of hedgehog but clear definition could not be made. The practical consensus at the moment for the phase IV validation, all images that image-analyzer captures and analyses will accept as genotoxic comet positive nuclei.
- VMT and Consultation team will prepare a color atlas for reference to distinguish between Comet and Hedgehog as soon as possible to send all participants for the phase IV before their starting analyses.

February 5

In vivo data analysis and next study plan (Chair: Dr. Uno)

1. Introduction - Dr. Uno summarized the outcome of phase II
   - Note: to correct 'Dunnett_2' described in the handouts to 'Unrestricted least significant different method'
   - There was some inconsistency of results among laboratories in the phase II study. Therefore, the phase III study was conducted using the revised protocol v.13.

Discussion about data acceptance criteria:

- ✓ Negative Control
  Liver 1-8%: reasonable based on the mean +/- 3SD
  Stomach 1-30%: too wide, and 6-20% seems reasonable based on the mean+/- 3SD in 4 labs
- ✓ Mean value should be in the range
- ✓ Positive control should be statistically positive [not to indicate the actual values]
- ✓ SD should be better than CV
- ✓ EMS 200mg/kg
- ✓ 2-fold or higher, 5 or higher and CV less than 50% in both liver and stomach

2. Data analysis of the phase III validation study

Dr. Uno summarized the phase III study

- Protocol version 13 was applied
- Chemicals: MNU (Chemical D), EMS (Chemical E), D-mannitol (Chemical F) + Positive control: EMS

Dr. Takashi Omori (Kyoto Univ.) explained the statistical analysis results

- Graphs in handouts reveal the mean value and the confidence interval instead of the S.E.
- All negative control groups showed the averages within the draft data acceptance criteria.
- All positive control groups were increased in the effects (difference and ratio) with statistical significance (t-test, $P<0.05$) which met the draft data acceptance criteria except for the effect (diff.) in the liver at Lab.4.
- MNU and EMS showed statistically significant results with the Dunnett test (both side, $P<0.05$) and the trend test ($P<0.05$) in all laboratories. D-Mannitol showed no statistical significance in all laboratories.

Discussion

- The reasons why magnitudes of the effects were different among labs? ==> Probably related to electrophoresis duration: Lab.1 20 min, Lab.2 30 min, Lab.3 20 min in liver & 30 min in stomach, Lab.4 15 min.

Comment to the acceptance criteria (Dr. Tice)

Keep the electrophoresis condition including 0.7V, 300mA, and duration (20 min) constant, positive control should be statistically positive (effect-difference). Negative control should be evaluated based on the laboratory's historical control database (at the beginning set guide number, e.g., 1-8% for liver and 3-20% for stomach). If the

data cannot meet the above tentative criteria, then the adjustment of the electrophoresis condition will be needed.

Positive control

Revise from one administration to two or three administration (24, 45h, or 0, 24, 45h) and sampling at 3h after the last administration using 3 animals instead of 5.

Set global cut off value for the positive response based on the analysis of the distribution of negative controls, e.g., mean + 2SD.

3. The selection of new laboratories to participate in the phase IV collaborative study.

Dr. Uno summarized the selection of new laboratories to participate in the phase IV. The 13 laboratories will join this validation effort including the leading laboratories.

4. Plan of the phase IV

- Purpose

Predictivity

Integration and/or combination

- Standard protocol version 14 (now preparing)

Three times administration at 0, 24, and 45 h and kill animals 3 h after the last administration. Positive control (EMS) will be also administered two times as same as test articles. (24 and 45 h and kill animals 3 h after the last administration (micronucleus (MN) in bone marrow will be detected in two times administration of EMS. If peripheral blood will be used, the 3 administrations (0, 24, and 45h) will be necessary).

The MN and Comet analysis in bone marrow/peripheral blood is just optional in this validation study.

The solid description about the condition of electrophoresis should be included in the protocol ver. 14.

Parameter: only % tail DNA but not tail length (should be defined, if used: tail migration or tail length by Comet IV) either Olive tail moment will be used. All data should be kept at each laboratory and only % tail DNA data should be submitted (keep the data of other parameter should be kept in the database for further use, if necessary).

- Delivery of chemicals is VMT matter (regional distribution may be considered).
- Common controls (one negative cont. + one positive cont.) are acceptable when two test chemicals are examined in one experiment.
- End of the validation study will be by the end of December, 2010.
- A workshop: image analysis, *e.g.*, comet *vs.* hedgehog.

  Now planning to hold a one (or two)-day workshop sometime in conjunction with the 10th ICEM 2009 - INTERNATIONAL CONFERENCE ON ENVIROMENTAL MUTAGENS, 8th International comet assay workshop or 7th World Congress on alternatives and animal use in the life sciences in August-September. At the workshop, technical questions by the participants will be also discussed. Data of the first experiment may be discussed if the data are available by the end of June (==>VMT matter? If chemical delivery by March then possible? Probably difficult due to both reasons of chemical delivery and lab acceptability)
- Some technical Q&A
- Two slides/tissue vs. three slides—>Two (Note: Data from Merck (two vs. three slides) should be analyzed)

Meeting documents

Meeting Document #1	International Validation Study of in *Vitro* Alkaline Comet Assay, *Responses provided by* Masamitsu Honma
Meeting Document #2	Data analysis and statically analysis of phase I study, *Responses provided by Masaya Suzuk*
Meeting Document #3	*Image analysis using Comet Assay IV, Responses provided by Madoka Nakajima*
Meeting Document #4	International Validation of the *in Vivo* Rodent Alkaline Comet Assay for the Detection of Genotoxic Carcinogens (Ver.14)
Meeting Document #5	In *Vivo* Comet Assay: Update on the on-going validation coordinated by JaCVAM, *Responses provided by Uno Yoshifumi*
Meeting Document #6	In *Vivo* Comet Assay: 3rd phase validation study, *Responses provided by Uno*

	Yoshifumi
Meeting Document #7	In *Vivo* Comet Assay: 4th phase validation study, *Responses provided by Uno Yoshifumi*
Meeting Document #8	Re2nd phase Figs and tables %tail DNA, *provided by Takashi Omori*
Meeting Document #9	3rd phase Figs and tables %tail DNA, *provided by Takashi Omori*
Meeting Document #10	In *Vivo* Comet Assay: Examination to select labs for 4th phase validation study, *Responses provided by Uno Yoshifumi*
Meeting Document #11	Memo for figs and tables on the phase 3 study

第7回コメットアッセイ国内委員会議事録

日　時：平成20年10月1日(水)14時～18時、10月2日(木)9時半～15時
場　所：大阪府　りんくうゲートタワー　501会議室
出席者：林、宇野、本間、浅野、森田、中嶋、小島（以上、両日）、鈴木、大森（10月1日のみ）
　　以上順不同、敬称略
配布資料：
1) Inter-laboratory validation for in vitro comet assay 1st validation study
2) Strategy for Chemical selection in the 4th Phase Validation study of in vivo comet assay
3) Candidate for the mail validation studies of Comet assay
4) Kirkland D. et al., Mutation Research 653(2008)99-108.
5) Participants of the 4th Phase Validation study of in vivo comet assay
6) Interim report on the consideration for participation in the international in vivo Comet assay validation study
7) コメットアッセイにおける個体の要約の指標に関して
8) International Validation of the In vivo Rodent Alkaline Comet Assay for the Detection of Genotoxic Carcinogens -Study Plan for 3rd Phase Validation Study-
9) International Validation of the In vivo Rodent Alkaline Comet Assay for the Detection of Genotoxic Carcinogens (Ver.13)
10) Chemical list of in vitro Phase II validation study of Comet assay
11) Phase II Validation Study of the In vitro Alkaline Comet Assay
12) Chemical list of in vitro Phase I validation study of Comet assay
13) Halosperm User's guide Manual del usuario, Biotech
14) Code Table of in vivo Phase II validation study of Comet assay
15) Chemical list of in vivo Phase II validation study of Comet assay
16) Science, Vol.321, 1144-1145.
17) Low data of in vivo comet assay
18) Candidate Chemicals for International Validation study of Comet assay

議題：

1．In vitroバリデーション結果と今後の方針

鈴木委員より、Phase Iバリデーション研究のデータの解析結果が資料1を用いて報告された。%DNA in tail、Tailモーメント、Tail長それぞれについて、被験物質毎に各施設の結果がまとめられ、その解析結果が報告された。

被験物質毎の結果から、EMSのバラツキが特定の施設で大きい、2-AAはS9mixの影響もあり適用濃度がばらつき、結果の解釈も異なる、TritonX-100では細胞毒性が認められる高用量でHedgehogと思われるコメットの増加が見られたなどが報告された。

これらの結果をもとに議論を行い、in vivoと同様の解析を行わない独立したバリデーション研究とすることを確認した。また、①In vivoでは個体間の比較を行うが、in vitroではtubeではなく細胞を単位とすること、%DNA in Tailの中央値を用いること（コメント：中央値と平均値のどちらが適当か、結論はでなかったと思います。どちらを選んでも良いので中央値（平均値だったような・・）を選ぶことにした、というニュアンスかと）、複数のtubeを用いることに大きな意味はないとされた、②細胞毒性として4時間処理後のTrypan blue染色における80%以上の生存細胞数、24時間処理後のrelative cell growth(RTG)では20%以上、その際のHedgehog頻度20%以下を細胞毒性の判断基準として、それら以内でコメットの増加を判断するデータの採用基準が明確にされた（PhaseⅡバリデーション研究のプロトコールに記載あり）。③陽性の判断基準としては、Dunnet解析ではなく、3濃度以上でバラツキを重みに

した回帰分析を行い、上向きの傾きにより有意差検定で判断する提案が大森委員よりなされた。④PhaseⅡバリデーション研究には陽性対照物質が設定されていないので、施設間差は陰性対照値と被験物質毎の傾きで判定するとことになった。以上の結果を受け、10 月中旬までにデータを解析し直した後、実行委員会および各施設にデータおよび報告書を本間 in vitro 委員長から提出することになった。

本間 in vitro 委員長より、in vitro 試験はベンツピレンやシクロフォウスファミドが陰性になるなど限界が明確という理由もあり、Phase Ⅲ以降のバリデーション研究の中止が示唆された。代案として、作用機構解明のための共同研究の提案がなされた。これに対して、現時点での in vitro バリデーション研究からの撤退は時期尚早との声もあり、複数の指標（小核や遺伝子突然変異）を組み合わせることなども視野に入れた来年度以降のバリデーション研究のテーマについて、本間 in vitro 委員長と小島委員で再考することになった。

2．In Vivo バリデーション

PhaseⅣバリデーション研究の参加施設について、資料 5 を用いて宇野 in vivo 委員長より報告がなされ、大森委員よりデータの解析結果が資料 17 を用いて報告された。15 施設から応募があったが、6 施設は辞退し、残り 9 施設のうち 7 施設を phase Ⅳバリデーション研究に参加可能と判断した。あとの 2 施設にはデータの追加提出を求めており、その結果を見て判断する予定。新たな参加希望があった 1 機関には他の参加機関と同要件を要求し、その結果を見て判断する予定。これまでに参加している既存 4 施設を含め、現時点では 13 施設の参加を予定していると説明された。

大森委員より、3 月の実行委員会において既存 4 施設にヒストリカルデータを依頼したが、集まっていないと説明され、データの収集は断念すると説明された。

解析方針について、資料 7 を用いて大森委員より説明がなされた。指標については、各個体の%DNA in tail の平均値を主体とするが、PhaseⅢバリデーション研究までは他の指標も解析し、その結果を見て phase Ⅳバリデーション研究では%DNA in tail のみに解析を絞るかを考えるとされた。陽性の判断基準としては、ratio と difference を用い、各々陰性対照の 2 倍と 5%以上、Dunnet と trend の両側検定 5%で判定することが提唱され、PhaseⅡバリデーション研究のデータを用いて検証することになった。これを 11 月末までに国際実行委員会で認証し，PhaseⅢバリデーション研究の解析に利用することとされた。なお、試験の成立基準は陰性対照値が肝で 1-8%、胃で 1-30%、陽性対照物質 EMS　200mg/kg が陽性の条件を満たす場合となっている。

ばらつきの原因として、解析ソフト“コメットⅣ”による細胞画像の取り込み方の差（どの細胞を計測するか）が指摘された。この問題を統一化するため、判定マニュアルを作成して、次回の国際実行委員会で統一化を図ることになった。マニュアルの作成は中嶋委員に委託された。

林委員長より、ICH の合意に従い、一般毒性試験にコメットアッセイを取り込む試みとして、14 または 28 日反復投与試験のトキシコキネティックスのためのサテライト群を用いて行う共同研究を国内では 3 施設で実施するとの説明があった。本共同研究にはJaCVAM プロトコール Ver.13が使われるとのこと。

一方、コメットと小核を同時に行うプロトコールの提案もなされていると説明され、PhaseⅣバリデーション研究のオプションに加える依頼が林委員長よりなされた。このプロトコールでは 2 回投与の 21 時間後、すなわち動物屠殺の 3 時間前に 3 回目の投与を行い、小核試験とコメットアッセイ両方に最適な投与スケジュールの元に標本を作製するものである。PhaseⅣバリデーション研究に向けて JaCVAM プロトコールを改良する必要がでたため、意見交換した。3 回投与でも結果に及ぼす影響は少ない、バリデーションの体勢にも影響は少ない、動物愛護を強調するためには致し方ないという受入れる意見の他に、こ

れ以上参加施設に負担をかけるべきではないとの否定的な意見もあった。PhaseⅣバリデーション研究ではオプションとして次回の国際実行委員会において最終的に参加施設に提案することとなった。

3．被験物質の選択

宇野 in vivo 委員長および森田委員の作成されたリスト（資料2、3および18）などを参考に、遺伝毒性や発がん性の有無、臓器特異性、特徴などを考慮の上、被験物質候補約 50 物質を選んだ。本リストをもとに、価格、納期を調査した上で次回の国際実行委員会までに最終リストを固めることが了承された。

4．国際実行委員会の日程

会場の下見を行い、本国際会議場を2月の国際実行委員会の会場とすることを確認した。また、以下の日程を確定した。

2009年2月4日終日　In vitro のデータ報告会および今後の計画について
2月5日終日　In vivo のデータ報告会
2月6日午前　判定マニュアル説明および今後の計画について
午後　実行委員会（VMT 会議？）

以降の予定、3月に被験物質配布、4月より in vivo PhaseⅣバリデーション研究開始、来秋国際実行委員会開催、2009 年内データ提出期限、2010 年夏シンポジウムおよびデータ報告会、2010 年末までに報告書作成

以上

第８回コメットアッセイ国内委員会議事メモ

日　時：平成21年2月3日(火)17時～19時
場　所：大阪府　関西空港ワシントンホテル　3階　珊瑚の間
出席者：林　真、宇野芳文、本間正充、浅野哲秀、森田健、中嶋圓、田中仁、鈴木昌也、山影孝司、中川ゆづき、小島肇、ワシントンホテル会場担当者　　以上順不同、敬称略

議事：

１．国際バリデーション実行委員会の日程確認

明日から開催される国際実行委員会の日程案を小島委員が紹介し、会議を円滑に進めるため、各自の役割分担について協力を要請した。要請を受け、会議の進行について意見交換がなされ、種々の提案がなされた。

２．国際実行委員会の資料確認

明日からの配布資料を配布し、資料の不備の有無を確認した。宇野委員および本間委員より資料の不足が指摘され、急遽印刷した。

３．会場係りとの段取り確認

会場の配置を指示し、会議を円滑に進めるため、明日からの３日間の段取りについてワシントンホテル会場担当者と相談した。さらに、追加資料の用意を依頼した。

以上