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.

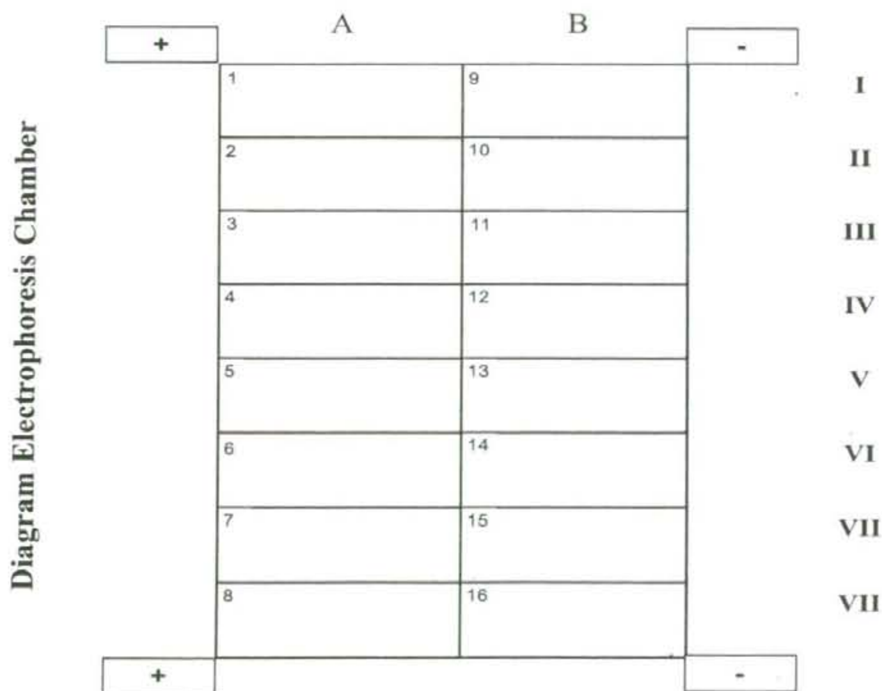
Olive PL, et al., Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cell using the "comet" assay. Radiat. Res., 122, 86-94, 1990.
Tice RR et al., Single cell gel/Comet assay: guidelines for in vitro and in vivo genetic toxicology testing. Environ. Mol. Mutagen., 35, 206-221,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



RED(+)

BLACK(-)

Position of slide in

International Pre-Validation Study of the In Vitro Alkaline Comet Assay
Version 5

PHASE II VALIDATION STUDY OF THE IN VITRO ALKALINE COMET ASSAY

Issued by the VMT (Masamitsu Honma)
Version 5, July 11, 2008

A. PURPOSE OF THIS DOCUMENT

This document is provided to conduct an international phase II validation study of the in vitro alkaline Comet assay. Following the phase I study, five leading laboratories conduct the in vitro Comet assay for 6 genotoxic or non-genotoxic chemicals to conform the in vitro Comet assay procedure and to make consensuses for evaluation and interpretation of the Comet results. In phase II study, we take blind trial. The management members review and validate the Comet results with the consultation of experts.

B. ORGANIZATION

1. Validation management team (VMT)
   M. Hayashi (JaCVAM/An-Pyo Ctr.), R. Corvi (ECVAM), M. Honma (NIHS), Y. Uno (MTP/MMS), L. Schechtman (Consultant), R. Tice (NIEHS/ICCVAM/NICEATM), H. Kojima (JaCVAM/NIHS; Secretariat)
2. Consultation team
   N. Asano (MMS/Nitto Denko), D. Lovell (Univ. of Surrey), T. Morita (NIHS), N. Nakashima (PMDA), Y. Ohno (JaCVAM/NIHS), T. Omori (Kyoto Univ.), YF Sasaki (Hachinohe Natl. Coll. of Tech.), M. Suzuki (An-Pyo Ctr.), S. Hoffmann (ECVAM), G. Speit (Unv. of Ulm), A. Collins (Unv. of Oslo), S. Park (KFDA), Y. Seo (Kyung Hee Univ.)
3. Leading laboratory
   K. Yamakage (FDSC, JP)
   B. Burlinson (HLS, UK)
   P. Escober (Boehringer-Ingelheim, USA)
   K. Pant (Bio-Reliance, USA)
   A. Kraynak (Merk, USA)

C. ASSUREANCE OF DATA QUALITY

The study will be conducted by the leading laboratories which have a facility of Good Laboratory Practice compliant (GLP).

D. TESTING PROCEDURE

1. BASIC PROCEDURES OF ALKALINE COMET ASSAY

We understand that the basic procedures for the Comet assay including cell lysis, un-winding, electrophoresis, neutralization, DNA staining, visualization, and Comet analysis are identical between in vivo and in vitro experiments. Those procedures were already established in the international validation study of the in vivo rodent alkaline Comet assay before. In this pre-validation study, the laboratory should conduct the Comet assay according to the established procedure. The summary of basic procedure is shown in Table 1.

Table 1

		In Vivo Comet Standard Procedure
Agarose gel and sample preparation	Bottom gel	1.0-1.5%-low-gelling temperature-agarose in PBS (if used)
	Sample gel (A)	0.5%-low-gelling temperature-agarose in PBS
	Solution of suspended cells (B)	Cells in HBSS with 20 mM EDTA and 10% DMSO*
	Mixture/ Final conc. of agarose	(A):(B)= 9:1/ 0.45%
Lysis and electroporation	Lysis solution	2.5M NaCl, 100mM Na2EDTA, 10mM Tris-base, 10% DMSO, 1% Triton-X (pH 10) *
	Lysys condition	Overnight, 4C
	Rinse solution/ Condition	Distiled water/ Dipping
	Electrophoresis solution	0.3M NaOH, 1mM EDTA (pH >13), <10C
	Electrophoresis condition	Unwinding 20min + Electrophoresis 0.7-1 V/cm (300mA), <10C
Staining	Neutralization/ Dehydrization	0.4M-Tris-base (pH 7.5) at least 5 min/ Absolute ethanol at least 5 min
	Staining dye/ Time	SYBR Gold/ 10 min
Scoring and statistics	Comet analysis	Comet IV, Tail length, Tail moment, % tail DNA

* DMSO and/or Triton X should be added just before use.

2. SPECIFIC ISSUES FOR THE IN VITRO ALKALINE COMET ASSAY; MATERALS AND METHODS

<u>2-1. Cells, cell lines</u>

The TK6 human lymphoblast cell line must be commonly used in the

pre-validation study. Other cells including human peripheral lymphocytes, L5178Y, V79, CHO, CHL/IU, or HepG2 can be used as a second choice if the laboratory prefers.

2-2. Media, cell culture condition, and cell stocks

Appropriate culture media, and incubation conditions (culture vessel, CO_2 and concentration, temperature should be used in maintaining culture. For TK6 cells, culture medium consists of RPMI1640 medium (GIBCO by Invitrogen Corporation; Cat. No.11875) supplemented with 200 ug/ml sodium pyruvate, 100 U/ml penicillin-100 ug/ml streptomycin (GIBCO by Invitrogen Corporation; Cat. No.15140), and 10 % (v/v) heat-inactivated fetal bovine serum (FBS).

The TK6 cells are always maintained in the culture medium at 37C in an atmosphere of 5% CO_2 and 100% humidity. Cell density is measured by a hemocytometer or an automatic cell counter and the cells are routinely diluted to ~2×10^5 cells/ml each day to prevent overgrowth (>1.5×10^6/ml).

The laboratory will thaw the delivered the cells and expansively culture in the medium and maintain approximately 1 week. Logarithmic growth is normally maintained with population doubling times of 11-15 h. The cell stocks should be made at approximately 1×10^6 cells/ml, 1ml/tube in culture medium containing 10% dimethylsulfoxide (DMSO).

Note) Each laboratory purchase TK6 cell line and FBS from ATCC by itself. ATCC can provide same lots of the TK6 cell (CRL-8015, lot#: 3296817) and FBS (30-2020, lot#: 6504229). TK6 can well grow with FBS and horse serum (HS), and the both conditions are available for any genotoxicity studies. However, the population doubling time with FBS is a little faster than that with HS.

2-3. Metabolic activation

Cells should be exposed to the test chemicals both in the presence and absence of the metabolic activation system (S9-mix). Each laboratory purchase S9 from MolTox (http://www.moltox.com/). Catalog number 11-105 (Post-Mitochondrial Supernatant: Sprague-Dawley rat liver. Male. Phenobarbital/5,6 Benzoflavone induced) must be commonly used in the study. The preparation of S9-mix and its use are as follows;

Energy source (co-factors):

6.7g NADP (disodium salt, mw = 787) and 12.5g isocitrate (trisodium salt,

mw = 258) are dissolved in a final volume of 500 ml distilled water and then filtered through a 0.2 micron filter to sterilize. Aliquots of this can be frozen at -70C for up to 2 years and used as needed (one use per aliquot).

S9-mix:

1.5 ml S9

6.0 ml energy source (from above)

Cultures:

Cell culture 9.15 ml

S9-mix 0.75 ml

Test chemical solution 0.1 ml

Final concentrations in culture media:

1 mM NADP

5.8 mM Isocitrate

15 ul/ml S9 (1.5%)

Note) In the absence of S9-mix, 150mM KCl should be used instead of S9-mix.

2-4. Duplicate cultures

The Comet assay for each chemical in the absence and the presence S9 should be conducted in duplicate, because the duplicate results will be appropriately evaluated statistically.

2-5. Duration of the treatment

The culture cells are treated with the chemical for 4 hours, and then the Comet assay should be immediately conducted. The 4 hours treatment is the optimal condition for in vitro Comet assay for most of cells.

2-6. Test chemicals and solvent

VMT selected 6 chemicals and delivered them to a chemical master in each laboratory. According to the direction of the chemicals master, the test chemicals should be prepared. The solvents used, in order of preference, are physiological saline, distilled water, or DMSO.

2-7. Evaluation of cytotoxicity

The three cytotoxicity tests should be commonly used for the studies.

1) Trypan blue dye exclusion test (TBDE) just after the treatment
2) Counting non-detectable cell nuclei (NDCN; hedgehog)
3) Relative cell growth for 24 h after the treatment

Optionally, other tests as follows could be taken if the laboratory prefers.

a) Late trypan blue dye exclusion test after the treatment

b) Relative survival (colony formation) just after the treatment

c) Neutral diffusion assay just after the treatment

d) Dual dye viability staining just after the treatment (Strauss et al., 1991)

e) Others (ATP concentration, mitotic index, etc.)

2-8. Top concentration and dose selection

The laboratory should conduct the Comet assay for the chemicals until 5mg/ml if no cytotoxicity is observed. When cytotoxicity is observed less than 5mg/ml, a top concentration is determined by cytotoxicity tests. The top concentration should show enough cytotoxicity, but not severe cell damage causing a lot of non-detectable cell nuclei (NDCN; hedgehog). The recommended top concentration is one with 80% TBDE, 20% NDCN, or no cell growth for 24 h after the treatment. The laboratory can conduct preliminary experiment for the dose-finding.

Each main experiment usually consists of one solvent control and at least five concentration of the test chemical. As a rule, 2-fold serial dilutions were prepared from the top concentration.

2-9. Standard procedure of treatment for TK6

i) The laboratory thaws the stoked cells into 50 ml of culture medium in a TS-75 culture bottle and starts cell culture. After several days, logarithmic growing TK6 cells are prepared approximately at 2 X 10^5 cells/ml with culture medium and divided into 15 ml plastic tubes by 9.15 ml aliquots, and 0.75 ml S9-mix (with metabolic activation) or 150mM KCl (without metabolic activation) are added into the tubes.

ii) The 0.1 ml of serially diluted chemical is added into the tube for starting treatment. The tube is closed tightly and incubated at 37C with gentle shaking on a rocker platform in an incubator for 4 hours.

iii) After the treatment, 1 ml of culture is taken into a new tube and is centrifuged at low speed (approximately 1,000 r.p.m. for 5 min), and supernatant is discarded. Each culture is washed with 1 ml of cold HBSS solution with 20mM EDTA and 10% DMSO, once by re-suspension and centrifugation. The cells are then re-suspended in 0.5 ml of the cold HBSS solution again. The cell suspensions are used for Comet slide preparation.

iv) After the treatment, a small portion of cell cultures are taken and

examined for trypan blue dye exclusion test.

v) The remained culture is also centrifuged at low speed (approximately 1,000 r.p.m. for 5 min), and supernatant is discarded. Each culture is washed with 5 ml of fresh medium once by re-suspension and centrifugation. The cells are then re-suspended in 10 ml of fresh medium and transferred to culture bottles (TS-25) or culture dishes. The cell density is measured by a hemocytometer or an automatic cell counter before starting culture. The cultures are incubated at 37C in a humidified incubator gassed with 5% CO_2 and in air. Twenty-four hours later, the cell density is measured again. The relative cell growth to the solvent control is calculated.

3. SLIDE PREPARATION

After the treatment, the cells are washed and made as single cell suspension with or without typsinazation. The cell sample for Comet assay should be finally suspended in cold HB solution with 20mM EDTA and 10% DMSO. The cell suspension sample and 0.5 % low melting agarose are mixed by 1:9 for preparing Comet slides.

E. DATA AND REPORTING

The data sheets in the phase II study will be sent later. After finishing all experiments, each laboratory will send the filled datasheets with final reports to VMT. The chemical master will also send the information of chemical name in the blind trial separately.

F. ARCHIVES AND REVIEW

The study reports and all of data should be retained according to the SOP in each laboratory. They will be provided to VMT for reviewing.

G. REFERENCES

Henderson, L., et al., The ability of the Comet assay to discriminate between genotoxins and cytotoxins. Mutagenesis, 13, 89-94 (1998)

Honma et al., Cytotoxic and mutagenic responses to X-rays and chemical mutagens in normal and p53-mutated human lymphoblastoid cells. Mutation Research 374, 89-98 (1997)

Honma, M., Hayashi M., Shimada H., Tanaka N., Wakuri S., Awogi T., Yamamoto K.I, Kodani N.-U., Nishi Y., Nakadate M. and Sofuni T. Evaluation of the mouse lymphoma tk assay (microwell method) as an alternative to the in vitro chromosomal aberration test. Mutagenesis, 14, 5-22 (1999).

Strauss, GHS., Non-random cell killing in cytopreservation: Implication for performance of the battery of leukocyte tests (BLT), I. Toxic and immunotoxic effects. Mutation Research, 252, 1-15 (1991)

Vock, EH., et al., Discrimination between genotoxicty and cytotoxicity in the DNA double-strand breaks in cells treated with etoposide, melpharan, cisplatin, potassium cyanide, Triton X-100, and gumma-irradiation. Mutation Research, 413, 83-94 (1998)

Comet: Osaka mtg.
Feb. 5, 2009

In Vivo Comet Assay: 4th Phase Validation Study

Facilities and Representatives of 4th Phase Validation Study

- Alphabetic order -

- AstraZeneca (UK) : Catherine Smith
- Bayer HelthCare (Germany) : Uta Wirnitzer
- BioReliance* (USA) : Buba Krsmanovic
- Covance (UK) : Lucinda Williams
- Food and Drug Safety Center* (JPN) : Kohji Yamakage
- Health Canada (Canada) : James P. McNamee
- Huntingdon Life Sciences* (UK) : Brian Burlinson
- Johnson & Johnson (Belgium) : Marlies De Boeck
- Merck* (USA) : Richard D. Storer
- Mitsubishi Chemical Safety Institute (JPN) : Kazunori Narumi
- Novartis Pharma (Switzerland) : Ulla Plappert-Helbig
- Sumitomo Chemical (JPN) : Sachiko Kitamoto
- The Institute of Environmental Toxicology (JPN) : Kunio Wada

* Leading laboratory

Purpose of 4th Phase Validation Study

The main purpose is to evaluate predictive capacity of *in vivo* comet assay against carcinogens.

In addition, when considering current discussion for ICH-S2 guidance, we should also consider to integrate *in vivo* comet assay into general toxicity studies or to combine it with other genotoxic endpoints such as micronucleus. We will focus on the combination with micronucleus assay in this validation study.

Draft: Outline and Schedule of 4th Phase Validation Study

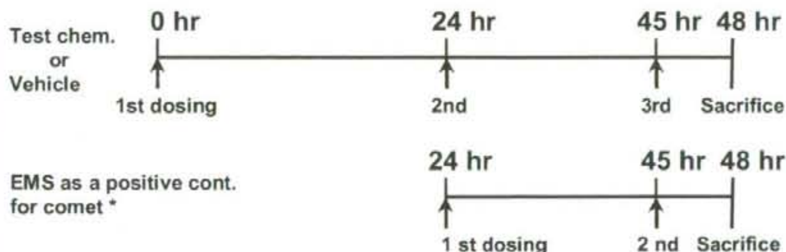
- ✓ The study will be performed in accordance with the version 14 study protocol and the study plan*. Please read them carefully.
  * This will be prepared for each validation study to define the purpose and specific points.
- ✓ Max. 4 coded test chemicals will be examined in each lab.
- ✓ Labs will have one year as a net experimental period to finish all experiments and submit the data spread sheet* to VMT. The clock of one year will start when you receive the test chemicals.
  * The sheet will be prepared by Omori-san, and Kojima-san will send it to each lab.
- ✓ Coded test chemicals, 25 grams or more each, will be sent in March-April from VMT to an individual within your lab who is not involved in this study.
- ✓ Basically, doses and vehicles for test chemicals will be determined in each lab based on a dose finding study, in case of no direction by VMT. MSDS will be enclosed with test chemicals, and you can refer to toxicity data such as LD50, if such data are available.

Draft: Outline and Schedule of 4th Phase Validation Study (cont.)

- ✓ The source and lot number of EMS, a positive control, will be informed by Kojima-san, and each lab will purchase it.
- ✓ Concerning the experimental design, you can chose such a modified design as two coded chemicals are examined in one experiment with one negative control group and one positive control group, i.e. total 8 groups in one experiment.
- ✓ The administration regimen to animals will be modified as shown in the following two slides.
- ✓ Other details on the study plan, e.g. how many chemicals will be examined, will be discussed in VMT mtg. held on Feb. 6., and then VMT will inform all participants of the details.
- ✓ The 4th phase validation study will hopefully finish by the end of 2010.

Modification of Administration Regimen

In order to enable to combine a comet assay with a micronucleus assay, three-times administration of test chemicals will be needed. Administration regimen will be change in the version 14 protocol as follows.



* A positive control for MN will be no longer required when considering current ICH-S2 discussion.

Points to be discussed related to the modification of administration regimen

- ✓Should we also examine MN in PB and/or BM in this validation study?
- ✓Should we also examine Comet in PB and/or BM in this validation study?

- ✓VMT will discuss above points in VMT mtg. held on Feb. 6 and decide how we shall do it.
  But, before the VMT discussion, I would like to hear your opinions about the acceptability of further works, i.e. Can you accept additional analysis of MN and Comet in PB/BM?.

Draft: Outline and Schedule of 4th Phase Validation Study (cont.)

- ✓ We are now planning to have a workshop of this validation study as one session of the International Comet Assay Workshop in August. At the workshop, the most important topic will be the image analysis, and we will explain how to distinguish between Comet and Hedgehog based on the discussion in this Osaka mtg.. We will invite all participants of this validation study to the workshop, especially who could not participate in this Osaka mtg..

- ✓ Labs that the representative has attended this Osaka mtg. will hopefully finish the first experiment by July. We will check the data quickly before the above workshop. If we feel necessity of discussion on the data, we will plan to have a mtg. at the workshop.

- ✓ Labs that the representative could not attended this Osaka mtg. will also start the first experiment. VMT will send a color atlas to instruct how to judge comet and hedgehog, and the labs should analyze the image in accordance with the instruction of color atlas strictly.

Steps of in vivo Comet Validation Studies
(Based on ECVAM Validation Process)

- ✓ Test definition
  - **Protocol optimization, Training ... 1st & 2nd phase validation**
  - **(Issues may remain in image analysis ... Workshop in Osaka mtg.)**
- ✓ Within-lab variability
  - **Total 6 exp. with EMS in each lab ... 2nd & 3rd phase validation**
- ✓ Between-lab variability
  - **· EMS data from 5 or 4 labs ... 1st, 2nd & 3rd phase validation**
  - **· 6 coded chem. data from 5 or 4 labs ... 2nd & 3rd phase validation**
- ✓ Predictive capacity
  - **Exp. with many coded chem. in 13 labs ... 4th phase validation**
- ✓ Reproducibility
  - **Will be evaluated with data from 1st, 2nd & 3rd phase validation**
  - **May be needed to check in 4th phase validation (to be discussed)**
- ✓ Transferability: **to be discussed, but**
  - **may be evaluated with data from lab selection study**
- ✓ Applicability domain: **to be discussed**
- ✓ Minimum performance standards: **to be discussed**
- ✓ Independent peer review: **to be discussed**

Blue: almost finished, Green: ongoing, Red: now planning

厚生科学研究費補助金（化学物質リスク研究事業）
「化学物質リスク評価法の国際的バリデーションに関する研究（H18-化学-一般-003）」

総合研究報告書

内分泌かく乱化学物質試験法のバリデーション

研究分担者　小野　敦　国立医薬品食品衛生研究所・総合評価研究室

研究要旨

本研究では、化学物質の内分泌かく乱性 in vitro 試験法のうち、行政的に有用であると判断された2種の試験法（HeLa 法および Lumi-cell 法）について、その信頼性や妥当性の検証を行うことを目的として、欧米の研究機関と共同で国際バリデーション研究を実施した。HeLa 法とは、我が国で開発された HeLa 細胞をベースとした ERαに対するレポーターアッセイ試験法であり、本研究班ではバリデーション結果をもとにOECDに提案されたガイドライン案に対するピアレビューコメントへの対応を行うとともに、ピアレビューにおいて要望が出されていたアンタゴニスト検出法のバリデーションを実施した。まず代表的化合物についてアンタゴニストアッセイを実施してバリデーションプロトコールを作成し、さらに標準物質の繰り返し測定により品質管理基準（Quality Control）及び性能基準（Performance criteria）を設定した。作成されたバリデーションプロトコールに従い、JaCVAM の主導により JaCVAM、ECVAM および米国 EPA のメンバーによるスタディーマネージメントチームのもと日本、欧州、韓国の施設における 3Task からなる国際バリデーション試験を実施した。バリデーション研究の準備段階である Task 1 では、アゴニストアッセイにおいて標準物質の測定を実施し、全てのラボで評価基準を満たす結果を得たが、アンタゴニストアッセイの準備段階である Task 2 においては、海外ラボでは標準物質の評価基準をクリア出来ず、原因究明のための追加検証が必要となった。一方、Lumi-cell 法は、米国で開発された ERαに対するレポーターアッセイ試験法であり、米国 ICCVAM で作成されたプロトコールをもとに欧州 ECVAM と JaCVAM の共同のもと日米欧の 3 施設の参加により、4Phase からなる国際バリデーションを実施した。Phase Ⅰ では、標準物質の 10 回繰り返し測定を行い、施設間での測定値に差はあるものの施設内変動は許容範囲内であることが示され、各施設における以降の測定のためのヒストリカルデータベースおよび品質評価基準の構築に成功した。Phase Ⅱ では、Phase Ⅰ で設定された品質評価基準に従い、アゴニスト・アンタゴニスト試験を各 4 物質について実施したが、多くの不採用データが出たことに伴い、品質評価基準項目の見直しを行った。引き続きアゴニスト、アンタゴニスト試験各 8 物質からなる Phase Ⅱb を実施した。その結果、特にアンタゴニスト試験では、多くの化合物で 3 施設における化合物評価結果が一致せず、追加試験の必要性が示された。

A. 研究目的

現在、内分泌かく乱性など新たな問題に対応すべく既存化学物質の安全性再評価が求められているが、対象となる化学物質の数は極めて多く、それらの安全性評価は予想以上に難しい。また、それらを従来の動物を用いる安全性試験法で評価するのは時

間がかかるだけでなく、経済性や研究資源の配分、動物福祉の面から問題であり、効率的かつ倫理的な新規試験法が求められ、多くの方法が開発されている。新規試験法について OECD では真に行政目的に合致し、国民の安全を確保するのに有用であるかを客観的に評価するために、バリデーションと行政的受け入れに関する基準を作成しているが、多くの試験法を我が国のみで開発するのは不可能であるし、OECD 基準を満たすバリデーションを行うことも容易ではない。

本研究では、OECD 内分泌かく乱物質評価タスクフォース（EDTA : Task Force on Endocrine Disrupters Testing and Assessment）により示されたコンセプチュアルフレームワークのレベル 2 に分類される化学物質の内分泌かく乱性 in vitro 試験法のうち、行政的有用性が推定される試験法として、我が国で開発された HeLa 細胞をベースにしたエストロジェン受容体 α（ER α）に対するレポーターアッセイ試験法（HeLa 法）および欧米で開発された同様のレポーターアッセイ法である Lumi-cell 法について欧米の研究機関と協力し、国際的なバリデーションを実施し、その妥当性を検討するとともに得られた結果から信頼性や再現性が確認された手法については、最終的に OECD ガイドラインとして提案することを目的として研究を行った。

B. 研究方法

B-1. hER α -HeLa-9903 細胞を用いた ER α アンタゴニストアッセイ法の再現性及び妥当性に関する研究

1. 試験施設

アンタゴニスト測定法再現性および妥当性検討のための測定は(財)化学物質評価研究機構・安全性評価技術研究所（試験責任者：武吉正博、赤堀有美、宮浦英樹）において実施した。

2. 実験材料および操作手順

1) 各被験物質は DMSO を用いて 10 mM となるようにストック溶液を調製し、4℃で保存した。

2) 溶媒対照（VC; Vehicle Control）：溶媒対照として、試験終濃度として 0.2%の dimethyl sulfoxide (DMSO、和光純薬）を含むウェル（6 連）を設定した。

3) 陽性対照：抗エストロゲン活性評価の陽性対象として、25 pM の E2 のエストロゲン活性を完全に抑制する 1 μM の OHT（3 連）を設定した。

4) 抗エストロゲン活性評価における基準活性：抗エストロゲン活性評価における基準活性として 25 pM の E2（6 連）を設定した。この濃度は前年度までの検討で、E2 自身の応答性が飽和しておらず、阻害のダイナミックレンジも狭くないと考えられた E2 自身の応答曲線から算出された EC80 相当濃度として採用した。

5) 細胞毒性評価用の陽性対照：DMSO に 100 μM の Digitonin (Dig、和光純薬)（3 連）を細胞毒性評価の陽性対象として設定した。

6) 細胞の応答性評価用の対照：抗エストロゲン活性評価における基準活性が E2 の最大活性ではない（つまり、E2 の応答性が飽和していない）ことを示す対照として、E2 の最大活性を誘導することができる 1 nM の E2（6 連）を設定した。

7) 暴露用被験物質あるいは対照物質の調製：EMEM-10%FBS 培地に DMSO で終濃度が目的濃度になるよう調製した被験物質あるいは対照物質を DMSO 終濃度が 0.2%となるように添加して調整した。

8) Luciferase Assay Reagent の調製：Luciferase Assay Substrate（プロメガ株式会社）の容器に付属の Luciferase Assay buffer 全量を直接加えて溶解し、-80℃で保存した。使用時には、必要量を解凍し 0.3 mM の MgCl2 を含む PBS と 1:1 で混和し、Luciferase Assay Reagent として使用した。

9) 測定手順：以下の手順に従って測定を行った。

① EMEM-10%FBS 培地を用いて HeLa-9903 細胞が 100 mm ティッシュに 70-90%コンフルエントになるように培養

② 細胞を測定用の 96-well プレートに播種（104 cells/100 μL/well）

③ CO2 インキュベータ内で培養（約 2 時間）

④ EMEM-10%FBS 培地で希釈した被験物質あるいは対照物質を 50 μL/well で添加

[プレートレイアウト]

	OHT			ICI			TAM			RAL		
	1	2	3	4	5	6	7	8	9	10	11	12
A	10^{-7} M + 25 pM E2			10^{-7} M + 25 pM E2			10^{-5} M + 25 pM E2			10^{-8} M + 25 pM E2		
B	10^{-8} M + 25 pM E2			10^{-8} M + 25 pM E2			10^{-6} M + 25 pM E2			10^{-9} M + 25 pM E2		
C	10^{-9} M + 25 pM E2			10^{-9} M + 25 pM E2			10^{-7} M + 25 pM E2			10^{-10} M + 25 pM E2		
D	10^{-10} M + 25 pM E2			10^{-10} M + 25 pM E2			10^{-8} M + 25 pM E2			10^{-11} M + 25 pM E2		
E	10^{-11} M + 25 pM E2			10^{-11} M + 25 pM E2			10^{-9} M + 25 pM E2			10^{-12} M + 25 pM E2		
F	10^{-12} M + 25 pM E2			10^{-12} M + 25 pM E2			10^{-10} M + 25 pM E2			10^{-13} M + 25 pM E2		
G	VC + 25 pM E2						1 μM OHT + 25 pM E2			100 μM Dig + 25 pM E.		
H	VC						1 nM E2					

⑤ CO2 インキュベータ内で培養（20～24 時間）

⑥ 培地の除去

⑦ Luciferase Assay Reagent の添加（50 μL/well）

⑧ 10 分間室温で静置

⑨ ルミノメータによる発光測定

3. 検討項目

1) 対照物質 E2 濃度の最適化確認

阻害対象となる対照物質 E2 の共存濃度によって ER アンタゴニストの示すレポーター遺伝子転写活性化の阻害効率に違いが生じることが想定されたため、E2 の共存濃度の最適条件を確認するために、化学物質と同時に添加する E2 濃度を終濃度 10 nM、6 nM、1 nM、600 pM、100 pM、60 pM、10 pM、6 pM 及び 1 pM でそれぞれ添加し、最適条件の検討を行った。測定物質には、4-Hydroxytamoxifen を用いた。

2) 再現性の確認

既に ER アンタゴニスト作用の判明している 4 物質 (Nafoxidine, 4OH-Tamoxifen, 4,4'-(Octahydro -4,7-methano-5H-inden-5-ylidene) bisphenol, RU-486)を用いて、独立した実験を 9 回実施して再現性を確認した。対照物質 E2 濃度は、25 pM にて実施した。測定に先立って常にルシフェラーゼを産生する細胞株(Control 細胞)のルシフェラーゼ活性への影響から実験を行う化学物質濃度域での細胞毒性を検討した。

4) 測定系の妥当性評価

事前スクリーニング以外で既に報告のある ER アンタゴニスト作用の判明している 3 種の化学物質及び ER アンタゴニストがないことが判明している 2 種の化学物質(Bisphenol A, Daizein)を用いて測定を行い、文献での結果と比較して測定系の妥当性に関して評価した。

5) アンタゴニストアッセイ法バリデーションのための品質管理基準 (Quality Control) 及び性能基準 (Performance criteria) 設定のための検討

抗エストロゲン活性が既知の 4 物質 (4OH-Tamoxifen, Tamoxifen, Raloxifen, ICI 182780)を用いてアンタゴニスト測定を 10 回繰り返して行い、得られた結果より評価基準値を算出した。

6) バリデーションプロトコールの作成および品質管理基準 (Quality Control) 及び性能基準 (Performance criteria) の設定

上記の検討結果からアンタゴニスト測定における標準物質を決定し、品質管理基準および性能基準を設定して、バリデーションプロトコールを作成した。

4. データ解析

各濃度区の発光強度の値から溶媒対照区 (VC) の平均値から差し引き、算出された 25 pM E2 の平均値でさらに各濃度区の値を除して 25 pM E2 に対する相対転写活性化倍率 (RTA; Relative Transcriptional activity (%)) を求めた。これら相対転写活性化倍率を用いて、以下の式より IC50 を算出した。IC50 値の算出には GraphPad PrismR Ver. 4 (GraphPad Software 社) を用いた。また、25 pM の E2 に対する RTA を 30 あるいは 50%抑制する濃度 (lin.IC30 あるいは lin.IC50) を求めた。

Y=Bottom + Top-Bottom)

/(1+10^((logEC50-X)*HillSlope))

HillSlope: **傾き**

X: **対数濃度** (M)

Y: **相対転写活性化倍率の抑制率** (%)

B-2. HeLa9903 細胞を用いたアンタゴニストアッセイバリデーション試験

1. バリデーション運営委員会 (Study Management Team (SMT)) の設置と参加施設

上記までの検討により設定されたバリデーションプロトコールおよび品質管理基準 (Quality Control) 及び性能基準 (Performance Criteria) をもとに多施設国際バリデーション試験を開始した。本研究ではバリデーション研究の透明性及び客観性を確保するため、Study Management Team (SMT)を組織し、実験の進行に関する情報の収集、解析及び状況に応じた指示を行った。SMT は小島肇博士(国立医薬品食品衛生研究所・新試験法評価室(JaCVAM))、小野敦博士(国立医薬品食品衛生研究所・総合評価研究室)、Miriam Jacobs 博士(ECVAM)、Susan C. Laws 博士(US-EPA)、寒水孝司博士(大阪大学)の 5 名で構成され、さらに菅野純博士(国立医薬品食品衛生研究所)及び OECD VMG non-animal メンバーをアドバイザーとした。実験技術面の支援に関しては本法の開発施設である化学物質評価研究機構安全性評価技術研究所(試験責任者:武吉正博、赤堀有美、宮浦英樹)をリードラボとして、試験計画書の作成、参加施設に対する実験指導、結果の解析、実験進行に伴う技術的問題点の把握及び問題解決のための技術支援を実施した。バリデーション試験参加施設は、本測定系を開発した化学物質評価研究機構をリードラボとして、欧州 ECVAM の推薦によりベルギーVITO および韓国 FDA の 3 施設とした。一方、本測定系は、これまで厚生労働省および経済産業省の共同により開発および検証が進められてきており、経済産業省において国内施設におけ