厚生労働科学研究費補助金(化学物質リスク研究事業)

分担研究報告書

遺伝毒性試験法コメットアッセイ(*in vivo*)のバリデーション

研究分担者　小島　肇　　国立医薬品食品衛生研究所薬理部新規試験法評価室室長
協力研究者　宇野　芳文　田辺三菱製薬株式会社研究本部安全性研究所第一部長
大森　崇　　同志社大学文化情報学部疫学・生物統計学研究室准教授
林　真　　食品医農薬安全性評価センターセンター長（副理事長）

研究要旨

本研究班ではコメットアッセイの国際的なバリデーション実行委員会を組織し、本試験の専門家とコンセンサスを取りながら、コメットアッセイの標準プロトコルの合意を目指してバリデーション研究を実施している。昨年度までに実施されたバリデーションの結果を解析し、良好な施設間再現性が得られることを確認できたことから、バリデーションPhaseⅣ-2として参加14施設（うち、国内4施設）の協力を得て、各施設が1～3物質を評価するバリデーションを実施している。

A．研究目的

遺伝毒性試験の中で、コメットアッセイとは単細胞ゲル電気泳動法とも呼ばれ、単離した細胞または核をアガロースに閉じ込めて融解した後、アルカリ処理で二本鎖DNAを単鎖にし、電気泳動による泳動パターンの変化によりDNA鎖切断などを検出する方法である。正常な細胞のDNAは非常に大きな分子であり、電気泳動してもほとんど移動せず、球形の核として観察される。一方、DNAで切断などが起こっている場合にはDNA断片の大きさに応じて移動し、球形の核を頭に尾を引いた彗星のような泳動パターンとなる[1)]。

本方法は、in vivoでもin vitroでも試験可能であること、細胞が得られるならばどのような臓器、器官でも試験可能であること、短期間で結果が得られること、初期のDNA損傷を検出できることから広く利用されている。しかし、研究室間の再現性を検証するバリデーションが実施されておらず、プロトコルの国際的な合意がなされてこなかった。このような課題を解決すべく、2008年度までに実施された「厚生労働科学研究　リスク研究事業」において、日本環境変異原学会/哺乳類変異原性(MMS)研究会、欧米の研究機関と協力して国際的なプレバリデーション（phaseⅢまで）を実施し、コメットアッセイの標準プロトコルを確定した。これまでの経緯を図1に示した。昨年度以降、さらにバリデーションを実施し、将来的にはOECDガイドラインへの掲載を目指すものである。

本年度(2010年度)は、バリデーションPhaseⅣ-2として参加14施設（うち、国内4施設）の協力を得て、各施設が1～3物質を評価するバリデーションを実施した。また、その過程で明らかになったコメットとヘッジホッグの判別方法の不統一を解消し、国際的な基準の統一を図るため、図解集（カラーアトラス）の完成を目指した。

B.研究方法

B-1 組織

本バリデーション組織は2006年度設立した。本年度は第8回国際バリデーション実行委員会（以後、実行委員会と記す）を2月にハンチントン（英国）で開催した。

1）国際実行委員会

委員長　林　真（食品医農薬安全センター：以下、安評センターと記す）

In vivo担当委員長

宇野芳文（田辺三菱製薬株式会社）

委員　L. Schectmann（前 Interagency

Coordinating Committee on the Validation of Alternative Methods : ICCVAM)
R. Tice (The NTP Interagency Center for the Evaluation of Alternative Toxicological Methods: NICEATM)
R. Corvi (European Centre for the Validation of Alternative Methods : ECVAM)

事務局　小島　肇（国立衛研　安全性生物試験研究センター　薬理部：Japanese Centre for the Validation of Alternative Methods : JaCVAM)

2）国内実行委員会

委員長　林　真

委員　宇野芳文
浅野哲秀（日東電工株式会社）
中嶋　圓（安評センター）
森田　健（国立衛研　医薬安全科学部）
本間正充（国立衛研　変異遺伝部）
小島　肇

3）コンサルタント

P. Escobar (Boehringer-Ingelheim)
D. Lovell (Univ. of Surrey)
大森　崇（同志社大学文化情報学部）
大野泰雄（国立衛研）

4）バリデーション参加施設

表1に示す14施設が参加した。参加している施設は、phaseⅣ-1で再現性の高い実験を行えることを確認できている。

B-2) 本年度の進捗

本バリデーションの目的は、化学物質の発癌性の予測性能を検討することである。バージョン14.2のプロトコルを用いて、参加14施設がphaseⅣ-2バリデーションを実施した。遺伝毒性発癌物質、非遺伝毒性発癌物質、遺伝毒性非発癌物質、非遺伝毒性非発癌物質で分類した40物質を各施設に配布し、1物質／施設、施設毎に1～3物質を評価するバリデーションを約1年掛けて実施した。

なお、%DNA in tailにおけるデータ採用基準を以下に示す。

陰性対照　肝臓の平均値　1-8%
胃の平均値　1-20%

陽性対照：EMS（エチルメタン酸スルフォネート）200mg/kg、経口2回投与、臓器を問わず、
溶媒との差　5%以上
溶媒との比　2倍以上

B-3) 被験物質の配布

被験物質の配布は、基本的に薬理部　新規試験法評価室で実施した。米国の参加施設については、NICEATMの支援を受けて配布した。被験物質コード番号は、A4201～A4240とした。

B-4) コメットアトラスの作成

コメット像分類の最終確認が実施され、テキストとして発行する準備を進めた。

（倫理面への配慮）

実験動物を用いる実験を伴うことから、各施設の倫理委員会の決定に基づき実験を行うように指示した。

C. 研究結果

C-1) 被験物質の配布

被験物質の配布については、本年度初めに配布した。一部物質の決定が遅れたこと及び連絡の不手際もあり、メルクとバイオリライアンスに各1物質が届いていないことが2010年末に判明した。これらは2011年2月中に送付した。

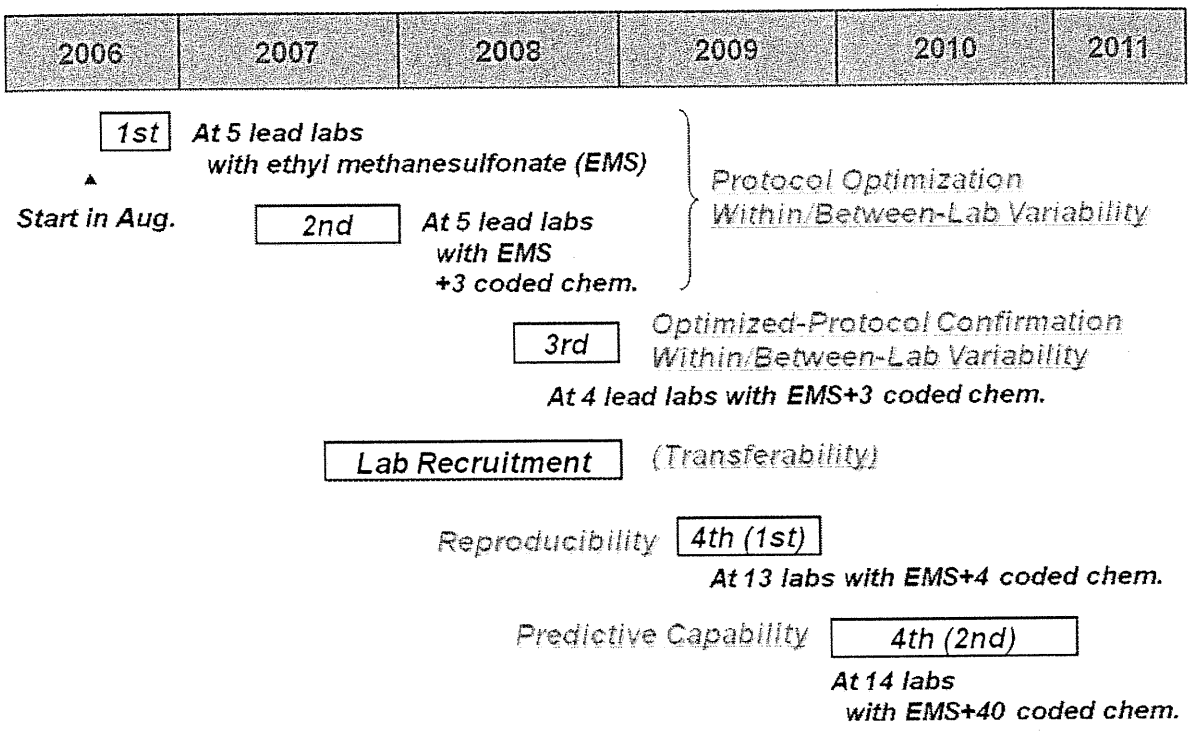

図１．これまでのバリデーションの経緯

表１．Phase IV-2 バリデーションの協力施設

施設名	国名	代表者
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	Hironao Takasawa
Novartis Pharma	Switzerland	Ulla Plappert-Helbig
Sumitomo Chemical	JPN	Sachiko Kitamoto
The Institute of Environmental Toxicology	JPN	Kunio Wada
ILS	USA	Cheryl A. Hobbs

*:Leading laboratory

C-2) データの収集

データは 2010 年夏頃より順次回収され、実行委員会が開催された 2011 年 2 月初旬までに 40 物質中 36 物質の結果が集まった。被験物質の配送不手際で集まっていない 2 物質の他に、一施設の実験が遅れた 2 物質があったためであるが、後者は 3 月初旬に実験が終了した。よって、38 物質の結果が収集できたことになる。これらのデータを統計学者がスクリーニングし、データを確定した。これまでに集まった結果のうち、陰性対照（肝臓、胃）の結果を、図 2 及び 3 に、陽性対照（肝臓、胃）の結果を、図 4 及び 5 に示した。なお、この値は溶媒との差である。

いずれもほとんどの結果がデータ受入れ基準を満たした。結果は示していないが、溶媒との比もすべての結果が 2 倍以上であった。図 2 に示すように、A4236 の陰性対照の％DNA in tail が基準の 1 ％より低かった。この理由は、電気泳動時間や泳動時の温度によらないと考察され、実行委員会は、同一施設でこの結果が繰り返されるようであれば、受入れ基準の変更も考慮すべきと考察した。その他のデータはすべて基準を満たしていた。

なお、データがすべて集まっていないこともあり、実行委員は、被験物質名のコード開示は時期尚早と判断した。そこで、コード非公開の上、第 8 回実行委員会で以下の議論がなされた。

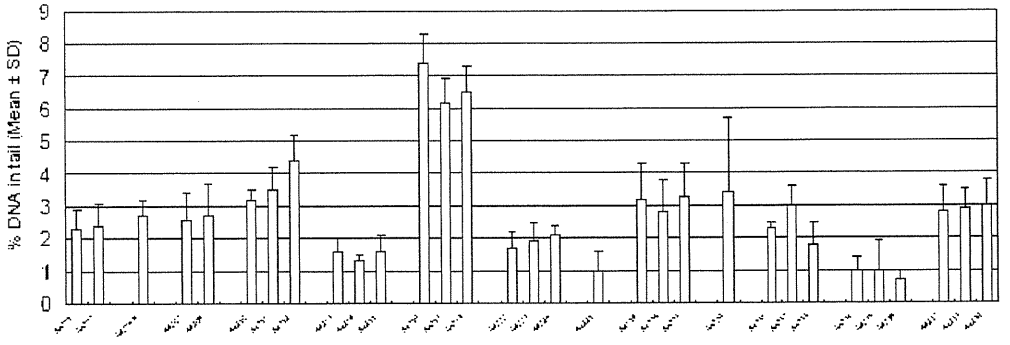

図 2．陰性対照値（肝臓）

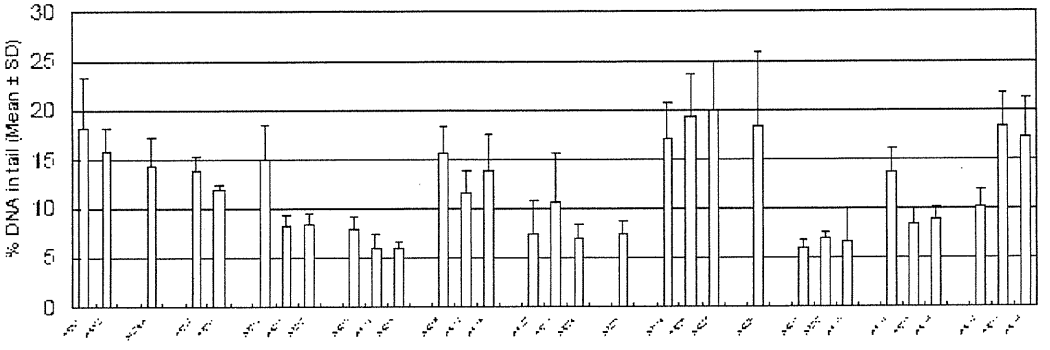

図 3．陰性対照値（胃）

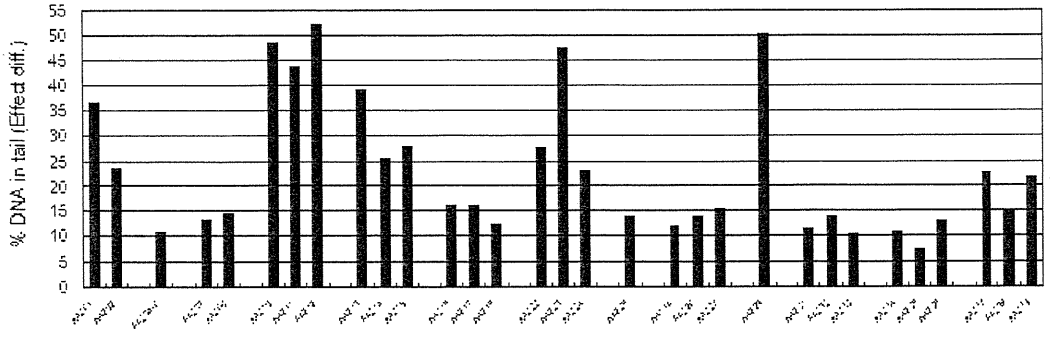

図４．陽性対照値（肝臓）

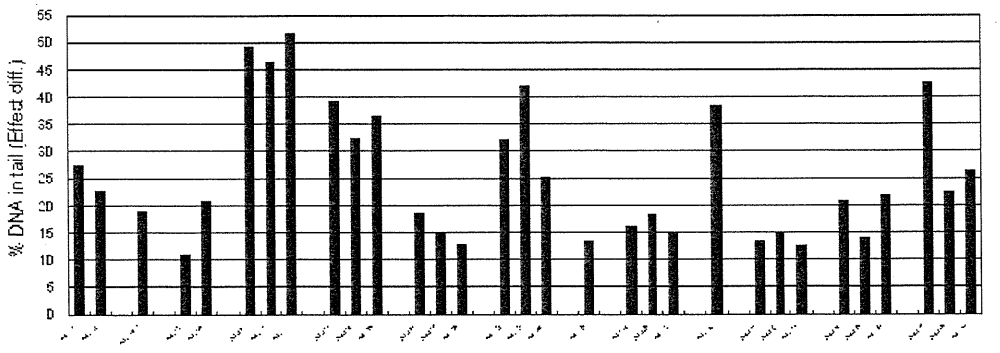

図５．陽性対照値（胃）

陰性対照及び陽性対照が受入れ基準を満たしていたことから、コード化された被験物質の解析が、実行委員会にて詳細に実施された。一部で、参加施設と実行委員会の判定が割れた。その理由は、ヒストリカルコントロールの扱いによるものであった。実行委員会は統計処理を基本にしている。実行委員会でのヒストリカルデータに関する討論の結果、プロトコルに従った統計処理の実施が確認された。データを吟味した結果、A4205 及び A4217 は非適合と判断された。最高投与量を溶解度限界で設定するなどしたため動物に毒性兆候がなく、陰性と判断できないとされたことによる。

一方、A4202, A4216, A4225 及び A4227 で観察された胃の%DNA in tail は有意に減少していた。これは細胞毒性によるものかもしれないとの指摘があった。そこで、それぞれの施設に、すべての濃度で胃の病理学的な検査を依頼した。

さらに、より有用なデータベースを作成するため、実行委員会は、陰性結果のすべての被験物質の最高濃度における肝臓と胃の病理検査を要望した。

いくつかの施設で、胃においてヘッジホッグが陰性対照群も含めて高率で確認された。胃のヘッジホッグは、表面を注意深くスクラバーで除去すると減少するとの説明が実験者よりなされた。

パラメーターに関して、メディアンとテールモーメントはコメットの結果の感度を高めるとの指摘があった。また、陰性対照値に対する fold-increase で判断する方が高感度との指摘もあったが、陰性対照値が低いときに誤った解釈を導く可能性があるとされ、推奨されなかった。

C-3) 追試験

A4205 及び A4217 は動物に毒性兆候が現れる最高投与量までの再試験を依頼することになった。A4211 は施設と実行委員会の判定が食い違ったため、A4219 は肝臓において実行委員会が判定不能としたため追試験実施となった。以上 4 物質の追加実験が必要と判断された。これらの被験物質は、同一コードで 3 月末までに同施設に再送され、追加実験が実施される予定である。なお、実行委員会は、2011 年 5 月末までにはすべての実験が終了するよう要望した。

C-4)その他

実行委員会は、phaseⅣ-1 及びⅣ-2 の報告書を各施設に 4 月末までに提出するよう依頼した。再試験の報告書も 8 月末までに提出が要望された。

実行委員会は、今秋までに全体のバリデーション報告書をまとめ、第三者評価の準備にかかると説明がなされた。また、これらの結果は、Mutation Research 特別号に投稿するとされ、小核試験の追加結果も含めた結果は各施設で投稿も可能と説明された。

C-5) コメットアトラス

コメット像分類の最終確認を進めてきた。画像の準備もでき、テキストとして発行する手筈を出版社と進めた。

D. 考察

ほとんどの施設の結果が、適合基準を満たしており、予想以上にまとまった結果であった。追試験も適合基準を満たさないからという理由ではなく、最高投与量や溶媒の選択から、陰陽性の判定が異なるかを判断するという目的に過ぎない。すべての結果が揃うことを後数カ月間待ちたいと考えている。集まったすべてのデータ収集を解析するため、2011 年 9 または 10 月に第 9 回実行委員会を日本で開催するとのアナウンスがなされた。

E. 結論

昨年度までに実施されたバリデーションの結果を解析し、良好な施設間再現性が得られることを確認できたことから、本年度は、バリデーションPhaseⅣ-2として参加14施設（うち、国内4施設）の協力を得て、各施設が1～3物質を評価するバリデーションを実施した。また、その過程で明らかになったコメットとヘッジホッグの判別方法の不統一を解消し、国際的な基準の統一を図るため、図解集（カラーアトラス）を完成させ、出版の準備を進めた。

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14）　小島　肇、桑原裕史、林卓巳、坂口眞由美、豊田明美、後藤　悠、中村恒彰、渡辺真一、阿彦恭子、大森　崇、音泉　卓、寒水孝、森本隆史、林　和彦、坂口　斉：眼刺激性試験代替法（STE試験）バリデーション研究　第3報、日本動物実験代替法学会第23回大会、東京（2010.12）

15）　小島　肇、北條麻紀：3次元培養表皮モデルを用いるコメットアッセイの条件検討日本動物実験代替法学会第23回大会、東京（2010.12）

16）　小島　肇：S5　化学物質の有害性評価に関する代替試験法開発―発癌性、発生毒性、免疫毒性－今後の展望、日本動物実験代替法学会第23回大会、東京（2010.12）

17）　小島　肇：培養皮膚モデルを用いた皮膚刺激性評価の現状、第10回ヒューマンサイエンス研究資源バンクセミナー、大阪（2011.1）

18）　小島　肇：動物実験代替法における国際動向、日本動物実験代替法学会・JaCVAM合同ワークショップ　動物実験の3Rにおける国際動向、東京(2011.2)

19）　小島　肇：皮膚細胞研究の応用とその可能性、日本化粧品技術者会大阪支部第15回勉強会　ワークショップ、大阪（2011.2）

20）　Kojima, H.: The Japanese Center for the Validation of Alternative Methods (JaCVAM): Recent ICATM contributions and Future Plans, Information Session: The International Cooperation on Alternative Test Methods (ICATM): Translating Science to Provide Improved Public Health Safety Assessment Tools, 50th Annual SOT meeting, Washington D.C.(2011)

21）　Kojima, H. and Hojyo, M.: Optimal conditions for performance of the comet assay using a three-dimensional human

epidermal model, 50th Annual SOT meeting, Washington D.C.(2011)

22) W Casey, P Ceger, F Deal, D Allen, G Clark, P Pazos, E Grignard, J de Lange, S Bremer, M Nakamura, H Kojima, A Ono, W Stokes.: Final Results of an International Validation Study of an *In Vitro* ER TA Test Method in BG-1 cells, 50th Annual SOT meeting, Washington D.C.(2011)

23) F Deal, W Casey, P Ceger, D Allen, C Yang, M Nakamura, H Kojima, A Ono, HJ Yoon, SY Ha[7], W Stokes: International Validation Study of an *In Vitro* Cell Proliferation Test Method for Screening Potential Estrogenic Agonists and Antagonists in MCF-7 cells, 50th Annual SOT meeting, Washington D.C.(2011)

24) J Kulpa-Eddy, R McFarland, R Isbrucker, M Halder, H Kojima, B Jones, NW Johnson, D Allen, E Lipscomb, S Morefield, W Casey, W Stokes: International Workshop on Alternative Methods to Reduce, Refine, and Replace the Use of Animals in Vaccine Potency and Safety Testing, 50th Annual SOT meeting, Washington D.C.(2011)

25) 小島　肇：日本における動物実験代替法の現状、シンポジウム S2H27　アジアにおける動物実験代替法の展開、第 84 回日本薬理学会年会、パシフィコ横浜（2011. 3）

26) 小島　肇：動物実験代替法の行政的受け入れと国際協調、シンポジウム S30 レギュラトリーサイエンスは社会にどう役立っているかー薬学系人材の役割と活躍の場を知るー、日本薬学会第 131 回年会、静岡（2011. 3）

G. 知的財産権の出願・登録状況
（予定を含む。）

1. 特許取得
なし

2. 実用新案登録
なし

3. その他
なし

J. 添付資料

添付資料 1： Minutes, 8th International Validation Management Team meeting in UK

添付資料 2：Protocol International Validation of the in vivo Rodent Alkaline Comet Assay for the Detection of Genotoxic Carcinogens (Version 14. 2)

添付資料 3：Study plan

Draft Minutes

The 8th Validation Management Team Meeting for the International Comet Assay Validation Study

Date: March 7, 2011, 2:00 p.m - 5:00 p.m.

Venue: Huntingdon Life Sciences, UK.

Attendees: Drs. Makoto Hayashi, Yoshifumi Uno, Masamitsu Honma, Raymond Tice, Raffaella Corvi, Takeshi Morita, Hajime Kojima, Jun-ichi Akagi

1) Dr. Uno asked the other VMT members their acceptability that Drs. Morita and Akagi joined this meeting because Dr. Morita was a consultation team member and greatly contributed to test chemical selection, and Dr. Akagi was a new secretariat of JaCVAM. All members accepted it.
2) Dr. Uno explained progress of the 4th phase-2nd step (4-2) validation study. Four chemicals were still being examined. Although Covance stained slides with ethidium bromide (EtBr), not SYBR gold, their data were considered acceptable because EtBr staining is generally used in comet assay. However, the validation study (control and treatment data) should be analyzed with their data included and excluded with a note of the protocol deviation.
3) Data acceptability was discussed using the summary file of negative and positive control data. In our data acceptance criteria, Effect (ratio), i.e., the fold-increase obtained by the positive control, strongly depends on the magnitude of the negative control, and a decision was made that this criterion would be deleted hereafter. Since the control data in all labs including Covance satisfied the primary data acceptance criterion, i.e., Effect (difference) between positive and negative controls is statistically significant with an absolute increase of 5% or higher, and almost all control data also satisfied the other data acceptance criteria, all of the data on the coded test chemicals were considered acceptable. However, the negative-call conclusion for A4205 were considered unacceptable because the highest dose level was selected based on the solubility limit of the compound in saline, in which the compound is relatively insoluble, rather than on using corn oil as the vehicle and basing dose selection on animal toxicity or 2000 mg/kg, whichever is lower. Data acceptability would be finally concluded after reviewing the data presented by each lab in the meeting tomorrow. During that review, we would need to carefully check on the basis for dose selection in tests where a compound appears to be negative for genotoxicity.

4) When summarizing the negative and positive control data in our validation report, the following points should be considered: a) a statistical analysis should evaluate for within-/between-lab variability and for potential differences in response depending on the vehicle used, b) the statistical analysis should be conducted including and excluding data from studies that deviated from the protocol or our data acceptance criteria, and c) the relationship between the negative control and positive control values in terms of absolute differences and fold increases should be compared. The method for statistical analysis will be discussed with our statisticians.

5) Comet assay results with 36 decoded test chemicals were discussed. In some cases, discordant judgment between the lab and the VMT was noted, e.g., A4219 was judged positive in lab but equivocal in VMT for the liver, A4211 negative in lab but increased %DNA in tail in VMT for the liver and A4205 negative in lab but equivocal in VMT for the liver. We would ask the labs the reasons for their conclusion at the meeting tomorrow. A4220 and A4238 were judged negative in labs due to severe cytotoxicity, but VMT simply called increased %DNA in tail based on statistical analysis results because we had not yet decided how histopathological results should be used in judging the biological relevance of comet responses. A4225, A4216, A4202 and A4227 were judged negative in labs but appeared to induce a significant decrease in stomach % tail DNA in VMT. As we could not find any published data indicating that those four chemicals are DNA cross-linking agents, the decreases might not be biologically relevant and the observed response a technical artifact. A4217, A4225, A4226 and A4234 were expected to be positive in the comet assay but were classified as negative; the reason for a negative call might be related to the target organ specificity of carcinogenicity. A4114 was negative, and this call was same as that in the 4-1 validation study. All comet assay positive compounds induced a positive response in the liver (i.e., there was no chemical detected as positive only in the stomach). Thus, investigating the stomach does not seem necessary, at least for the chemicals tested.

6) If histopathology is applied to organs showing negative results in comet assay, more useful database can be established. We will ask participants about the feasibility of doing additional histopathology at tomorrow's meeting. Another idea is that in order to reduce scorer bias, one lab evaluates the histopathology for all available organs from all labs.

7) VMT discussed which chemicals should be retested. The following were

candidates for retests: A4205 due to the lack of toxicity in animals when tested up to 100 mg/kg, and A4219 due to equivocal responses in VMT judgment for the liver. The same lab should reexamine the same chemical.

8) VMT discussed when the test chemicals could be decoded. Decoding of test chemicals including the chemical category information should be done after all data (at least data-spreadsheets) are submitted to VMT.

9) Dr. Honma explained that all data of the 3rd phase in vitro study were collected. He proposed to decode chemicals and disclose the data at the 8th meeting. All members accepted it.

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

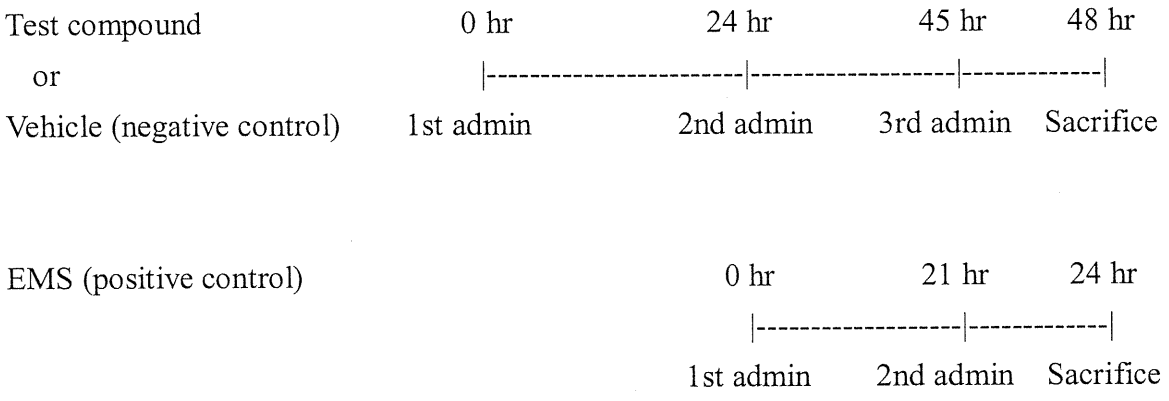
Compound	Dose (mg/kg/day)	Number of animals (see note 1)
Vehicle (negative control)	0	5
EMS (positive control)	200	5
Test compound	Low (1/4 of high)	5
Test compound	Medium (1/2 of high)	5
Test compound	High*	5

*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.



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 can not 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

3.1.1. 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 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.

3.1.3. 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; 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.

3.1.3.2. 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; 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