第6回コメットアッセイ国内委員会議事録

日　時：平成20年2月28日（火）15:00-18:30

場　所：国立衛研　センター会議室

出席者：大野泰雄、林　真、浅野哲秀、宇野芳文、山影康次、中嶋　圓、森田　健、本間正充、大森　崇、
鈴木雅也、中川ゆづき、上田摩弥、小島　肇、L. Hothorn (Leibniz Univ.)
以上順不同、敬称略

配布資料

1） Statistical Evaluation of the in vivo Micronucleus Assay
2） 第5回コメットアッセイバリデーション国内委員会議事録
3） PhaseⅡ配布物質の資料
4） Draft Analysis Report for comet assay 2nd pre-validation study (version 0.1)
5） Figs and Table 2008/02/22
6） JACVAM第1回ワークショップおよび1st International Workshop
7） コメットアッセイバリデーション実行委員会関連スケジュール
8） 参加者旅程
9） 予備試験　配布物質資料
10） 予備試験参加施設リスト
11） Progress/Plan/schedule of in vivo comet assay
12） SD、SEと分散

議事：

1．Professor L. Horthonのセミナー

オブザーバーとして参加されたProfessor L. Horthonより、資料1に示す"Statistical Evaluation of the in vivo Micronucleus Assay "に関する講演がなされた。小核試験の統計学的評価法として、DunnettやWilliam法を用いた評価指標について説明を受け、質疑応答を行った。

2．In vivo解析結果について

小島委員より、資料3を用いてPhaseⅡバリデーションの物質コードの開示がなされた。物質Aが2,6-Dinitrotoluene、Bがacrylamide、Cが2,4-Dinitrotolueneであると報告された（後日、確認済み）。

次に、大森委員よりPhaseⅡバリデーション解析結果の説明の前に、資料12を用いて「SD、SEと分散」の言葉の定義について説明があった。これをもとに、資料5を用いて解析結果を把握してほしいと説明された。

資料5を用いた解析結果の説明として、肝臓でも胃でも、陽性対照物質EMS (Ethylmethanesulfonate)を用いた施設内の平均のバラツキを100とすると、施設間は300-400であったと説明された。この数字は妥当であるが、このバラツキが施設間0～444と極端であり、施設間差は極めて大きいと考察された。このバラツキのため、あるいはEMSの反応性が低いためか、胃の場合、物質Cの試験時の陽性対照が2施設で統計学的に陰性と判断され、プロトコールの手法にまだ解決すべき問題があることが明確になった。

PhaseI バリデーション結果との比較を求める意見もあったが、 EMS の 1 回のみの結果であり、比較は無意味との意見が示された。指標としては、3 指標の中で%tail DNA には上限があり、わかりやすいという意見が多かった。

また、明らかに陽性となると判断されて選ばれた物質 B において、濃度依存的な反応が不明確な施設があり、被験物質選択者の予想を裏切る結果となったと宇野委員長よりコメントが示された。データの中で、物質 B の 1 施設の最高濃度が 200mg/kg である点については、小島委員が提出データから濃度を再確認することになった。

これらの問題について、3 月の実行委員会（Validation Management Team：VMT）会議で議論するため、本資料と開示表をバリデーション実行委員会はもとより、すべての関係者に事前に配布するものの、施設コードは開示しないことが林および宇野委員長の判断で決まった。なお、大森委員より、次の課題は acceptable criteria であると説明された。

3．3 月シンポジウムおよび VMT 会議の予定について

資料 6～8 を用いて、3 月 11 日のワークショップおよび 3 月 12～14 日の VMT 会議の予定について小島委員より説明がなされ、参加者の予定確認が行われた。ワークショップにおいては、Dr. Andy Kraynak にプロトコールで未解決の問題点（電気泳動の温度、細胞毒性の病理標本作製の可否）について講演を依頼するよう要望が出された。できれば、Dr. Brian Burinson や Dr. Patricia Escober にも問題点について講演をお願いしたいと宇野実行委員長から依頼があった。

VMT 会議では 3 月 12 日に in vitro、3 月 13 日は in vivo、3 月 14 日は午前中に追加討議を行い、遅くとも昼食後散会することが確認された。In vitro 結果については、本間委員長が参加施設に呼び掛け、現状までの結果を集めると説明された。ただし、In vivo で議論すべき内容が多いとの指摘を受け、in vitro の検討が終わり次第、12 日からも検討を始めることになった。

4．今後の予定について

小島委員より、資料 9～10 は PhaseⅢバリデーションに参加するため、JaCVAM から送付した被験物質を用いて、予備試験を実施中の施設が紹介された。

今後の計画、予定については、3 月の VMT 会議で議論されることになった。

以上

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ばら、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dish当りではなく細胞毎に行うこと、%DNA in Tailの中央値が適当である、複数のdish数には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%の細胞数、24時間処理後の相対的な細胞数では20%減少、その際のHedgehog頻度20%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。9 施設から応募があったが、2 施設は辞退、2 施設にデータの再提出および新たな希望があったベリンガーマンハイムにデータを要求していると説明された。これまでに参加している既存 4 施設を含め、12 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 の平均値を用いることにするが、PhaseⅢバリデーション研究までは他の指標も解析するとされた。陽性の判断基準としては、ration と difference を用い陰性対照の 2 倍、Dunnet 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。試験の成立基準は陰性対照値が 1－8％、陽性対照物質 EMS　300mg/kg が陽性の条件を満たす場合とされた。

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、1 4 または 28 日反復投与のトキシコキネティックスとともに行う共同研究を 3 施設で実施していると説明があった。本共同研究には JaCVAM プロトコール Ver.13 が使われているとされた。

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 ICH のメンバーから受けていると説明され、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 3 時間前に 3 回目投与を行い、両方の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）および Dr.Karkland の最近の論文（資料 4）をもとに、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 物質を絞り込んだ。本リストをもとに、価格、納期を絞り込ん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　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　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

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

Minutes

International Validation Kick off Meeting On Comet Assay

Date: August 14-15, 2006
(August 14, 9:30 to 17:30, August 15, 9:00 to 12:00)
Venue: Sapporo Koseinenkin kaikan,
Sapporo, Japan

Participants:
M. Hayashi(NIHS), T. Hartung(ECVAM), L. Schectman(FDA), R. Tice(NICEATM), Y. Uno(Mitsubishi Pharma Co.), Y. Uno(Mitsubishi Pharma Co.), H. Kojima(JaCVAM, NIHS)
N. Asano(Nitto Denko Co. : MMS president), N. Asano(Nitto Denko Co. : MMS president), B. Burlinson(Huntingdon), M. Honma(NIHS), D. Lovel(Univ. of Surrey), T. Morita(NIHS), N. Nakashima(OECD), N. Nakashima(OECD),
P. Clay(Syngenta, CTL), P. Escoba(Invitrogen), R. Storer(Merck), M. Nakajima(Anpyo-Center), K. Yamakage(FDSC),
Y. Nakamura(LSG Co.: observer),

1 Welcome address

Dr. Kojima called the kick off meeting on comet assay and everyone introduced themselves. After that, he checked the materials in advance or immediately distributed for this meeting.

Dr. Ohno, who has grant for this validation study, welcomed everyone to the meeting on comet assay in Sapporo. After greeting, the following agenda items were discussed.

2 Overview of the pre-validation study

Dr. Hayashi presented the circumstances and the overview of the pre-validation study on comet assay that he used power point manuscript "***International validation study of in vivo & In vitro Comet assay***".

3 Validation process

Dr. Tice presented what is test method validation that he used the latter half of power point manuscript "***Pros and Cons of the Comet Assay for Human Risk Assessment***" and"***Comet assay validation: modular approach***".

4 Process for OECD guideline

Dr. Nakashima presented the overview of OECD , mutual acceptance of data, test guidelines and Good Laboratory Practice that he used power point manuscript "***OECD Activities on Chemical Safety***".

5 Other information

Dr. Hartung presented the other information that he used manuscripts "***Comet assay and cell array for fast and efficient genotoxicity testing***" proposal by COMICS in EU.

6 Selection of validation management team chair

The members were unanimous in their approval of Dr. Hayashi as the chairperson in the validation management team.

7 Object of this validation

Dr. Hayashi discussed the proposed purpose that he used Word file manuscript "***JaCVAM initiative International validation on in vivo and in vitro comet assay***". The purpose determined 1) To clarify some technical aspects and to recommend the standard technical procedure of this assay. 2) The intra- and inter-laboratory reproducibility of this assay will also be evaluated.

8 Structure of validation team and responsibilities

Dr. Hayashi discussed the proposed organization and responsibilities of all members that he used Word file manuscript "***JaCVAM initiative International validation on in vivo and in vitro comet assay***". He confirmed Merck will not attend pre-validation study as lead laboratory.

9 Protocol issues

Dr. Hayashi discussed the proposed the protocol issues that he used Word file manuscript "***TESTING PROCEDURE OF IN VIVO ALKALINE COMET ASSAY FOR THE INTERNATIONAL VALIDATION STUDIES(DRAFT, VER. 3)***".

9-1 GLP

This study will be conducted under the spirit of Good Laboratory Practice in each GLP compliance laboratory.

9-2 Positive control

Ethyl methanesulfonate (EMS) will be used as a positive control. All laboratories used same butch of EMS ad Invitrogen use EMS and MMS.

9-3 Negative control (solvent/vehicle)

When no instruction from the management team, an appropriate solvent/vehicle will be chosen for each test substance by each testing facility from the following ones: physiological saline, 0.5%w/v sodium carboxymethylcellulose aqua solution, corn oil.

9-4 Test animals and size of study,

The following conditions were determined; Rat: Crl: CD (SD), male, at the time of dosing: 7-9 weeks of age, 5 animals/group raised in national regulation.

9-5 Preparation of reagent solution

Following solutions will be prepared freshly for use and used within one week. The conc. of agarose gel is 1.0% (w/v) for the bottom layer and 0.5 % (w/v) low-melting agarose gel. The staining solution consists of Cyber Gold.

9-6 Administration to animals and sampling

The test substance will be usually administered twice to animals orally by gavages. Animals will be anesthetized with ether or a proper anesthetic at 3 hours after dosing, and the liver and the stomach.

9-7 Experimental design

Unknown chemicals will be tested 2 or 3 dose, and EMS at 250mg/kg. The Management team recommends Benzo[a]pyrene(as mild mutagen), 2,6-Diaminotoluene (as week mutagen).

9-8 Isolated nuclei vs whole cell

The method each testing facility should use to obtain single cells.

9-9 Slide preparation, electrophoresis, staining

The nucleotides will be left to be unwinded for 20 minutes. After alkali unwinding, the slides will be electrophoresed for X minutes from 0.7 to 1 V/cm, with accompanying amperage of 0.25-0.30 A. 5-10 % migration.

Electrophoresis solution should be maintained a constant temperature at 25 +/- 2 C and low temperature. To confirm the liquid temperature changes, the liquid temperature of at least three points will be measured and recorded at the start of alkali unwinding, the start of the electrophoresis, and the end of electrophoresis.

9-10 Comet visualization and analysis

Fifty cells –migrating image per slide will be analyzed. Two slides per point will be used.

9-11 Endpoint and analysis (including IA *vs* categorization)

Dr. Yamakage presented the endpoint that he used power point manuscript "***Comparative Data on Image Analyzer for Comet Assay***". **As** the endpoint, the Percentage of DNA in tail will be calculated using an image analyzer system.

9-12 Cytotoxicity (histopathology *vs* others)

Dr. Tice recommended the Neutral diffusion assay. Only when a positive result in comet analysis is obtained in a tissue, histopathology will be examined for the tissue according to the SOP in each testing facility.

10 Data collection

Dr. Hayashi on behalf of Dr. Omori presented the data collection that he used power point manuscript "***About data sheet ver.1.0 for our validation study***".

11 Time schedule proposal

Dr. Hayashi discussed the proposed time schedule proposal that he used Word file manuscript "***JaCVAM initiative International validation on in vivo and in vitro comet assay***". Dr. Hatrung commented that this assay is incomplete to start a validation study. Prior to start the pre-validation study, he insisted on optimizing a protocol in this assay. Therefore, each laboratory will pre-test for pre-validation using above protocol (chemical: EMS only) at September-November, 2006. In the beginning of December, next meeting will be held in Tokyo, Japan. JaCVAM will prepare the venue, schedule and budget for the next meeting.

12 *In vitro* comet assay

Dr. Honma presented the proposal of *in vitro* study that he used power point manuscript "***The in vitro Comet assay-Study Plan-***". This validation starts next year.

Abbreviation

Anpyo-center	Biosafety Research Center, Foods, Drugs and Pesticides
ECVAM	European Center for the validation of alternative methods
FDA	Food and Drug Administration
FDSC	Food and Drug Safety Center
Huntingdon	Huntingdon Life Sciences
Invitrogen	BioReliance,invitrogen bioservices
JaCVAM	Japanese Center for the validation of alternative methods
MMS	Mammalian Mutagenicity Study
Merck	Merck Research Laboratories
NICEATM	Interagency Center for the Evaluation of Alternative Toxicological Methods
NIHS	National Institute of Health Sciences
OECD	Organisation for Economic Co-operation and Development

Draft Minutes

The 2nd International Validation Meeting on Comet Assay

Date: December 10-11, 2006
(December 10, 9:00 to 17:30, December 11, 9:00 to 12:00)
Venue: Tokyo University Komaba Campus,
Tokyo, Japan

Participants:
M. Hayashi (NIHS), T. Hartung (ECVAM), L. Schectman (FDA), R. Tice (NICEATM), Y. Uno (Mitsubishi Pharma Co.), H. Kojima (JaCVAM, NIHS), N. Asano (Nitto Denko Co.: MMS president), B. Burlinson (Huntingdon), P. Escobar (Invitrogen), M. Honma (NIHS), A. Kraynak (Merck), Y. Nakagawa (FDSC), M. Nakajima (Anpyo-Center), M. Ueda (Anpyo-Center), K. Yamakage (FDSC), Y. Ohno (NIHS), Y. Nakamura (LSG Co.: observer), N. Tanaka (FDSC: observer)

1 Welcome address

Dr. Ohno, who has grant for this validation study, welcomed everyone. Dr. Hayashi asked everyone to introduce himself or herself. After that, the following agenda items were discussed.

2 Data of the pre-validation study

Drs. Burlinson, Escobar, Kraynak, Yamakage and Nakajima presented each testing data of this pre-validation study with EMS. Participants felt that overall testing results were well validated. Dr. Escobar also reported results in a co-laboratory comparison study on the comet image analysis with SYBR Gold staining. There seemed no obvious difference among laboratories.

3. Protocol issues: discussion and conclusion

Protocol issues were discussed item by item on the version 10 protocol, and a revised version 11 protocol was prepared (see a MS word file **"protocol V11"**). Points to discussion were as follows: 1) need to add EDTA into low-melting

agarose in order to keep lower background (issued by FDSC): additional examination will be conducted in Invitrogen and HLS, 2) lost gel problem in unwinding and electrophoresis process under room temperature (issued by HLS), 3) should studies be conduct under GLP or just in GLP compliant laboratories?, 4) need to detect cross-linker type mutagens (issued by Dr. Tice), 5) the number of animals per group: need to analyze with power calculation after validation, 6) an average DNA migration in a negative control group: 1-15%? of % DNA in tail, 7) dehydration problem (issued by HLS), 8) recognition of hedgehog: >90% of % DNA in tail?, 9) need to conduct histopathology in validation studies, 10) statistics.

4. Time schedule proposal

Dr. Hayashi confirmed the time schedule in near future as follows: 1) each leading laboratory will send a EXCEL data sheet and an individual study protocol of this pre-validation study to Dr. Kojima ASAP, 2) Dr. Omori will analyze the data by the end of January, and then Dr. Hayashi will share the results with all participants (by the middle of February?), 3) Dr. Uno will send a protocol check sheet of this pre-validation study to leading laboratories, all testing laboratories will fill in each item column and then send it to Dr. Kojima by the end of this year, 4) Drs. Hayashi and Uno will revise a standard draft-final protocol for the further validation studies by the end of this year.

VMT discussed the future validation study plan in a VMT meeting this morning but could not decide yet. Points should be discussed for the future plan are as follows: 1) test compound selection considering chemical classes, 2) the total number of test compounds to reach a conclusion, 3) recruitment and selection of testing facilities, including technical transfer to new entries, 4) design of validation studies, 5) animal reduction, especially for positive/negative controls. Dr. Hartung commented that a lean design is preferable for validation studies, 20 test compounds, for example, are too small to calculate the sensitivity and specificity of a comet assay, and one resource to evaluate the performance without many new data may be to mix up the existing data. VMT will continue to discuss about above points thorough e-mail/tele-conference, and then inform all participants of a draft plan and ask them the feasibility.

5. *In vitro* comet assay

Dr. Honma presented a proposal of an *in vitro* comet assay protocol. A kick-off meeting (and a workshop) on the validation will (may?) be held on May 2007

together with a cell-transformation assay meeting.

Abbreviation

Anpyo-center	Biosafety Research Center, Foods, Drugs and Pesticides
ECVAM	European Center for the validation of alternative methods
FDA	Food and Drug Administration
FDSC	Food and Drug Safety Center
Huntingdon	Huntingdon Life Sciences
Invitrogen	BioReliance, invitrogen bioservices
JaCVAM	Japanese Center for the validation of alternative methods
MMS	Mammalian Mutagenicity Study
Merck	Merck Research Laboratories
NICEATM	Interagency Center for the Evaluation of Alternative Toxicological Methods
NIHS	National Institute of Health Sciences
OECD	Organisation for Economic Co-operation and Development
VMT	Validation Management Team

Draft Minutes

The 3rd meeting for

Validation Management Team on International Comet assay validation studyComet Assay

Date: August 26, 2007, 9:00 a.m.-2:30 p.m.

Venue: Hotel East 21,
Tokyo, Japan,

Participants:
M. Hayashi (NIHS), T. Hartung (ECVAM), L. Schechtman (Consultant), R. Tice (NIEHS), Y. Uno (Mitsubishi Pharma), H. Kojima (JaCVAM, NIHS), N. Asano (Nitto Denko: MMS president), R. Corvi (ECVAM), P. Escobar (BioReliance), M. Wind (CPSC), A. Rispin (EPA), T. T. Morita (NIHS), M. Y. Nakagawa (FDSC), M. Nakajima (Anpyo-Center), T. Omori (Kyoto Univ.), S.N. Park (KFDA), Y.F. Sasaki (Hachinohe Tech. Coll.), M. Ueda (Anpyo-Center), K. Yamakage (FDSC), S. Tonomori (LSG)

Discussion:

1. Statistical analysis plan for second *in vivo* pre-validation study
   Firstly, Dr. Omori explained how to use EXCEL data sheets provided for the second *in vivo* comet assay pre-validation. There are 3 sets of input columns for each animal, but to input data into 2 of 3 columns is acceptable. Anyone does should not change any format provided. Total 3 chemicals were examined in the second *in vivo* comet assay pre-validation. One of them, VMT directed the all dose levels, but other 2 chemicals should be found the optimal dose levels in each laboratory. The laboratory should select dose levels by their way referring to the protocol version 12, section 1.4.1. To share the information how to select the dose levels, each laboratory will report the reason why he/she chooses the top dose. Such information would be helpful to determine the criteria for the

dose selection in the main study, and Dr. Escobar will provide a reporting format (tabular format?).

Secondly, Dr. Omori explained a statistical analysis plan for the second *in vivo* comet assay pre-validation. The objectives of main validation studies are to investigate reproducibility, and robustness and validation of effect. The group agreed that the estimate (unit) is an animal but not a cell. Primary measures are mean untransformed % tail DNA in the liver of each animal and that in the glandular stomach of each animal. Secondary measures will be also reported, i.e. mean untransformed tail length, mean untransformed Olive tail moment, and median untransformed % tail DNA. Data analysis will be done on assay sensitivity, intra-laboratory variability, comparisons between the dose groups and the vehicle group, inter-laboratory variability, correlation analysis for parts, investigation on the reduction in the number of animals, and using three endpoints for judgment. Regarding Object 2, acceptable criteria on a range of variation of effect will be discussed in the next meeting. Pair-wise tests may produce false positive results, and multiplicity should be also considered for the comparison between the dose groups and the vehicle group. As the 2 chemicals not directed the top dose by VMT may be applied as different dose levels among laboratories, the dose effect should be considered for the slope analysis on estimate.

Regarding judgment, there were many opinions as follows: 20% increase of mean % DNA may define positive (cut-off value); each laboratory reports the own each laboratory call on the positive/negative judgment (put new space for the laboratory call); cut-off value + statistical confidential interval; we will fix the cut-off point later; biological significance *vs.* statistical significance; and sensitivity and specificity will be discussed based on the actual data.

2. IntermissionMember of the VMT

We welcomed Dr. M. Honma as an additional member of VMT under the condition that he and his laboratory do not attend at the validation study (VMT issue). Dr. T. Hartung was replaced by Dr. R. Corvi. Then new members of VMT for the comet assay are Drs. R. Colvi, M. Hayashi, M. Honma, L. Schechtman, R.R. Tice, and Y. Uno. The group also agreed that Dr. R. Tice represents NICEATM and also ICCVAM.

3. Progress, schedule and plan of *in vivo* validation study

Dr. Uno explained progress of the 2nd pre-validation study and the draft schedule and plan for the main validation studies.

Regarding recruitment of main validation studies, 5 laboratories have already applied to join the project. We need to discuss following issues: about the exception for participants

(VMT issue); how many laboratories should be participated in the main studies; how many chemicals should be examined in the main studies (20 may not be enough to make final decision); and how many chemicals/laboratory = is *e.g.* 4 chemicals/year practically possible (laboratory issue)?

We need to evaluate the assay validity with both data from the on going validation studies and the published studies (retrospective validation), *i.e.*, prospective and retrospective validation. We need to compile and review such published data. However, the current protocol is not fit to the old data, and we should determine acceptable criteria for the old data, *e.g.*, to check conformity to published recommendations (Tice, et al., 2000, Hartmann, et al., 2003, Burlinson, et al., 2007).

Regarding the main studies, there were opinions as follows: if inconsistent data are obtained from two laboratories that evaluate a same chemical, what should we do?; the current trial will be enough to show the reproducibility, and if the second present validation study goes well, then one chemical per each laboratory may be sufficient to evaluate the assay sensitivity/specificity; we can test twice chemicals, which may be è much more important. Regarding acceptability of new laboratory (historical data + one chemicals testing), VMT members discuss in the luncheon VMT meeting.

Again, how many chemicals should we evaluate? (VMT issue)? To examine the assay specificity is more important (use of in vitro +ve non-carcinogens), and not so many typical genotoxic agents will be included. Practical selecting chemical selecting should be s è based on the modes of action. COLIPA has a chemical list for assay validation, and we should may refer to it.

Regarding possibility of incorporation with general toxicity studies (considering ICH S2(R)), we need to think about it after the main validation studies. It may be enough to use very limited number of chemicals to be evaluated for the purpose. REECH activity is also affective

4. Luncheon VMT meeting (member: Hayashi, Hartung, Schechtman, Tice, Uno)

VMT members discussed how to select laboratories for the main studies. For examination, 2 chemicals, i.e., +ve and –ve chemicals, should be tested in a candidate laboratory. For less experienced laboratories, dose-response tests on the +ve control chemical (EMS) should be tried twice to confirm the reproducibility, and then +ve and –ve chemicals should be tested as for experienced laboratories. Technical transfer is important and we need to seek the possibility to have it at three regions.

For in vitro comet validation, we recommend to use two kinds of cell line.

5. Protocol of *in vitro* pre-validation study

Dr. Uno reported the summary of in vitro comet assay meeting on August 24 (see the meeting minute). Assay sensitivity of *in vitro* Comet assay was discussed again, and we need to continue the discussion. Following are candidates of leading laboratories: Anpyo, BioReliance, FDSC.

At the luncheon VMT meeting, members recommended to use two kinds of cell lines for the in vitro comet assay validation study.

Minutes

The first meeting for

international *in vitro* Comet assay validation study

Date: August 24, 2007, 3:30-5:30 p.m.

Venue: Hotel East 21,
Tokyo, Japan,

Participants:
M. Hayashi (NIHS), M. Honma (NIHS), L. Schectman (Consultant), R. Tice (NIEHS), Y. Uno (Mitsubishi Pharma), N. Asano (Nitto Denko: MMS president), T. Morita (NIHS), N. Nakashima (PMDA), T. Omori (Kyoto Univ.), P. Clay ( ), P. Escobar (BioReliance), M. Nakajima (Anpyo-Center), M. Ueda (Anpyo-Center), K. Yamakage (FDSC), Y. Nakagawa (FDSC), Y. R. Seo (Kyung Hee Univ.)

Discussion:

1. *In vitro* Comet assay protocol

   When reviewing published or available protocols, they seem overall comparable. A protocol for the international *in vitro* Comet assay validation studies will be basically same as that for *in vivo* Comet assay except for some specific points *in vitro*, e.g. cell preparation method.

2. Low sensitivity of *in vitro* Comet assay

   When reviewing *in vitro* Comet assay data, the assay seems less sensitive compared to the TK mutation assay and the MN assay, as extremely high doses are required to obtain positive results. But it may not be a major issue, because the sensitivity would depend on many factors such as cell types used, experimental conditions, and how to investigate cytotoxicity. This point should be discussed further more.

3. Purpose of validation study

To optimize the study protocol in the pre-validation phase. Finally to investigate linkage to *in vivo* Comet assay results (biological relevance). Need to examine the same chemicals investigating in *in vivo* Comet assay validation (and more chemicals should be examined *in vitro*). Test chemicals should be selected from following four categories: genotoxic carcinogens, genotoxic non-carcinogens, non-genotoxic carcinogens, and non-genotoxic non-carcinogens. After confirmation of robustness of the study protocol and usefulness of the assay (replacement of CA assay and/or MLA assay?), we should work on OECD to establish the guidance for regulatory use. Need to be clarified how to use this assay in regulatory aspects.

4. Items in study protocol

4-1. Cell type:

Primarily, TK6 cells.

Secondarily, mouse lymphoma cells, human lymphocytes, CHL cells, etc.

Comments: Comparison among cell types should be necessary.

Immortal cell lines having normal p53 status would be preferable.

Adhering cell lines vs. suspended cell lines, which is better?

TK6 cell line: easy to induce apoptosis (cf. WTK1: p53 mutant).

Exposure duration, dose range, S9 exposure: sort term exposure does not matter for necrosis/apoptosis.

When and how should cyototoxicity be evaluated? After 24 hr exposure or soon after 3-4 hr exposure?

How many cell lines should be examined? It may depend on numbers of chemicals examined.

An idea is to examine same chemicals with two (or more) cell lines in two (or more) different study groups.

It may be needed to hear worldwide scientist's opinions, which cell lines are preferable for Comet assays.

In pre-validation phase, use of one cell line may be acceptable.

Cell lines used should be from same resource and same passage number.

4-2. Test chemical selection:

Need to be examined with/without S9-mix conditions.

Use of fewer chemicals would be sufficient for optimization of the study protocol.

Following five chemicals will be examined (chemical names will not be coded)

EMS : same as *in vivo* positive control; without S9-mix

MMC : cross-linker; expecting a negative result; without S9-mix

Cycloheximide : non-genotoxic and cytotoxic agent; without S9-mix

Triton X : non-genotoxic and cytotoxic agent; without S9-mix

2AA: with S9-mix

4-3. Treatment time: 4 hrs

4-4. Cytotoxic parameter

When and how should cytotoxicty be checked?

Dye exclusion test with trypan blue: possible to examine immediately after exposure period. This is 1st choice in the pre-validation study (although sensitivity seems bad).

Relative cell growth: 24 hrs after the treatment. 2nd choice.

Relative survival: immediately after exposure period. 2nd choice.

Neutral diffusion assay: immediately after exposure period. 2nd choice.

4-5. Number of samples: duplicate cultures/dose will be used.

4-6. Dose level: at least 5 doses including negative control will be observed.

4-7. Lysis and electrophoresis: basically same as *in vivo* Comet assay conditions.
Room temp in electrophoresis, OK? Need to be examined.

4-8. Statistics: to be discussed.

5. When will this pre-validation study start?
To be discussed.

6. Leading labs selection
To be discussed.
Many EU companies are interested in *in vitro* Comet assay, and thus they will hope to join.
(Leading labs in *in vivo* Comet validation may also be leading labs in *in vitro* Comet validation)

Minutes
4th meeting of the International Validation study
On Comet Assay
Date: March12-14, 2008
(March 12, 9:00 to 17:00, March 13, 9:30 to 17:00, March 14, 8:30 to 15:00)
Venue: Atagawa hights,
Shizuoka, Japan

M. Hayashi (NIHS), Y. Uno (Mitsubishi Tanabe Pharma Co.), M. Honma (NIHS), H. Kojima (JaCVAM, NIHS), N. Asano (Nitto Denko Co. : MMS president), T. Morita (NIHS), M. Nakajima (Anpyo-Center), K. Yamakage (FDSC), Y. Nakagawa (FDSC), J. Tanaka (Anpyo-Ceneter), M. Ueda (Anpyo-Center), T. Omori (Kyoto Univ.), A. Kimura (NIHS), N. Tanaka (FDSC), A. Sakai (FDSC), B. Burinson (Huntingdon Life Science), P. Escober (Boeringer-ingelheim), D. Lovel (Univ. of Surrey), A. Kraynak (Merck), Young Na Yum (KFDA), R. Corvi (ECVAM), S. Hoffman (ECVAM), K. Pant (BioReliance)

1

March 12, 2008/Day 1/In vitro comet 1

- This section was leaded by Dr. M. Honma
- Dr. Hayashi addressed welcome remarks and Dr. Kojima gave hose keeping announcement.
- Dr. Honma leads today
- Dr. Honma showed his data (his laboratory is not a leading laboratory)
  - S9 problem
  - Treatment time was not critical robust for the treatment time
    - 4 h had no problem
  - Cytotoxicity
    - Trypan blue and RCG
  - Comet assay itself is as same as for in vivo assay
    - Duplicate culture to show the reproducibility

2