Animal Use in the Life Sciences, Rome (2009)

12) Kojima, H., Matsui, T., Kohara, A., Yoshida, A. and Nakamura, Y.: GCCP initiatives in Japan, 7th World Congress on Alternatives & Animal Use in the Life Sciences, Rome (2009)

13) Ono, A., Takeyoshi, M., Bremer, S., Jacob, M., Laws, S., Sozu, T. and Kojima, H.: The International validation study for the ER alpha STTA antagonist assay using HeLa9903, 7th World Congress on Alternatives & Animal Use in the Life Sciences, Rome (2009)

14) Allen, D., Deal, F., Ceger, P., Gordon, J., Pazos, P., deLange, L., Bremer, S., Nakamura, M., Kojima, H., Ono, A., Tice, R. and Stokes W.: Testing of coded substances for a multi-phases international validation study of an estrogen receptor (ER) transcriptional activation (TA) assay, 7th World Congress on Alternatives & Animal Use in the Life Sciences, Rome (2009)

15) Kojima, H., IIjima, M., Matsunaga, K., Sasa, H., Itagaki, H., Okamoto, Y., Nishiyama, N., Mita I., Washida, J., Masuyama, K., Onodera, H., Masuda, M., Ohno, Y.: Review of an alternative to animal testing for safety evaluation of cosmetic ingredients using Quasi-drug, 7th World Congress on Alternatives & Animal Use in the Life Sciences, Rome (2009)

16) Wind, M., Blakey, D., Kojima, H., Kreysa, J. and Stokes, W.: What is the international cooperation on alternative test methods (ICATM) and what is its role?, 7th World Congress on Alternatives & Animal Use in the Life Sciences, Rome (2009)

17) Kojima, H.: JaCVAM's role in the 3Rs and ICATM, 7th World Congress on Alternatives & Animal Use in the Life Sciences, Rome (2009)

18) Kojima, H.: Recent progress and future directions at JaCVAM, 7th World Congress on Alternatives & Animal Use in the Life Sciences, Rome (2009)

19) Inoue, T., Masuda, M., Akita. M., Kojima, H. and Ohno, Y.: JaCVAM statement on new alternative to animal testing, 7th World Congress on Alternatives & Animal Use in the Life Sciences, Rome (2009)

20) Takeyoshi, M., Kojima, H., Omori, T., Sozu, T. and Yoshimura, I.: Validation study for non-radioisotopic local lymph node assay based on BrdU incorporation (LLNA-BrdU), 7th World Congress on Alternatives & Animal Use in the Life Sciences, Rome (2009)

21) Kojima, H., Ando Y., Yamaguchi Y., Kosaka T., Suzuki T., Yuasa A., Watanabe Y., Shinoda S., Idehara K., Yoshimura I., Miyaoka E., Ishiyama K., Kato M., Omori T.: Validation of LabCyte EPI-MODEL24, an *In Vitro* Assay for Detecting Skin Irritants, 7th World Congress on Alternatives & Animal Use in the Life Sciences, Rome (2009)

22) Yamamoto, N., Hirano, K., Kato, M., Hata, K., Horiguchi, M., Taniguchi, K. and Kojima, H.: Cell surface maker of corneal epithelium stem cells and culture, 7th World Congress on Alternatives & Animal Use in the Life Sciences, Rome (2009)

23) Lowther, D., Wind, M., Stokes, W., Barroso, J., Zuang, V., Amcoff, P., Kojima, H., Prinsen, M., Tice, R., Allen, D. and McCall, D.: International acceptance of in vitro alternative ocular safety testing methods: the isolated chicken eye (ICE) test method (Draft OECD TG 438), 7th World Congress on Alternatives & Animal Use in the Life Sciences, Rome (2009)

24) Merrill, J., Wind, M., Stokes, W., Barroso, J., Zuang, V., Amcoff, P., Kojima, H., Jacobs, A., McCall, D. Allen D. and Tice, R.,: International acceptance of in vitro alternative ocular safety testing methods: bovine corneal opacity and permeability (BCOP) test method (Draft OECD TG 437), 7th World Congress on Alternatives & Animal Use in the Life Sciences, Rome (2009)

25) Hayashi, M., Uno, Y., Honma, M., Schectmann, L., Tice, R., Corvi, R., Morita, T., Asano, N. and Kojima, H.: In vivo Comet Assay: Update on the on-Going international validation study, 7th World Congress on Alternatives & Animal Use in the Life Sciences, Rome (2009)

26) Honma, M., Kojima, H., Morita, T., Uno, Y., Asano, N., Nakajima, M., Corvi, R., Tice, R., Schectman, L. and Hayashi, M.: International validation study of the in vitro alkaline comet assay, 7th World

Congress on Alternatives & Animal Use in the Life Sciences, Rome (2009)

27) Kojima, H., Arai S. and Hojo M.: Adequate conditions for performance of comet assay using 3-dimensional human epidermal model, 7th World Congress on Alternatives & Animal Use in the Life Sciences, Rome (2009)

28) Stokes, W., Wind, M., Matheson, J., Jacob., A., Casati, S., Kojima, H., Allen, D., Burns, T., Salicru, E., Strickland, J. and Tice, R., Internationally harmonized performance standards (PS) for the murine local lymph node assay (LLNA), 7th World Congress on Alternatives & Animal Use in the Life Sciences, Rome (2009)

29) Honma, M., Kojima, H., Morita, T., Uno, Y., Asano, N., Nakajima, M., Corvi, R., Tice, R., Schechtman, L. and Hayashi, M.: International validation study of the in vitro alkaline comet assay, 25th ICEM, Florence (2009)

30) Uno, Y., Kojima, H., Honma, M., Schechtman, L., Tice, R., Corvi, R., Asano, N. and Hayashi, M.,: In vivo Comet Assay: Update on the on-Going international validation study, 25th ICEM, Florence (2009)

31) 小島　肇：動物実験代替法における国内外の動向、日本薬学会関東支部大会、埼玉（2009）

32) 小島　肇：In vitro 安全性･機能性評価及び作用メカニズム･新規物質探索研究の最前線 、第 22 回動物細胞工学シンポジウム、東京（2009）

33) 小島　肇：医薬部外品の承認申請における安全性に関する資料のあり方検討会、日本産業皮膚衛生協会　秋季研修会、京都（2009）

34) Kojima, H.: Japanese views in the 3Rs in the 21st century, ZEBET's 20th Anniversary Symposium, Berlin, Germany (2009)

35) Kojima, H.: Organization of JaCVAM and its activity, KoCVAM International Symposium and 6th Congress of KSAAE, Seoul (2009)

36) Kojima, H.: Utilization of an alternative to animal testing for safety evaluation of cosmetic ingredients using Quasi-drug, The 17th ICDS (International Contact Dermatitis Symposium) and the 10th APEODS (Asia-Pacific Environmental and Occupational Dermatology Symposium), Kyoto (2009)

37) Kojima, H.: Japanese approach to regulatory acceptance of new skin sensitization testings with considerations to animal welfare and 3Rs, The 17th ICDS (International Contact Dermatitis Symposium) and the 10th APEODS (Asia-Pacific Environmental and Occupational Dermatology Symposium), Kyoto (2009)

38) 小島　肇、安藤洋子、山口能宏、小坂忠司、鈴木民恵、湯浅敦子、渡邉幸彦、篠田伸介、出原賢治、吉村　功、宮岡悦良、石山賢也、加藤雅一、大森崇：培養皮膚モデル LabCyte EPI-MODEL24 を用いた皮膚刺激性試験代替法のバリデーション研究、第 22 回日本動物実験代替法学会総会・学術大会、大阪（2009）

39) 小島　肇、飯島正文、松永佳世子、佐々　斉、板垣　宏、岡本裕子、西山直宏、小野寺博志、見田　活、鷲田　淳、益山光一、増田光輝、大野泰雄：医薬部外品の承認申請における安全性に関わる資料のあり方検討委員会報告、第 22 回日本動物実験代替法学会総会・学術大会、大阪（2009）

40) 小島　肇、井上　達、増田光輝、秋田正治、大野泰雄：動物実験代替法公定化のための JaCVAM 提案書、第 22 回日本動物実験代替法学会総会・学術大会、大阪（2009）

41) 小野　敦、武吉正博、Susanne Bremer、Miriam Jacob、Susan C. Laws、寒水孝司、小島　肇：HeLa9903 細胞を用いたエストロゲン受容体転写活性化試験によるアンタゴニスト検出法の国際バリデーション、第 22 回日本動物実験代替法学会総会・学術大会、大阪（2009）

42) 本間正充、山影康次、Burlinson, B.、Escobar, P.、Pant, K.、Kraynak, A.、林　真、中嶋圓、鈴木雅也、Corvi, R.、宇野芳文、Schechtman, L.、Tice, R.、小島　肇：In vitro アルカリコメットアッセイ国際バリデーション研究、第 22 回日本動物実験代替法学会総会・学術大会、大阪（2009）

43) 小島　肇、笠松俊夫：IWGT 報告　トピックス３：予測性の高い in vitro 試験の提案、日本環境変異原学会第 38 回大会、清水・静岡（2009）

44) 中嶋圓、小島　肇、宇野芳文、本間正充、林　真：コメットアッセイの国際バリデーション、日本環境変異原学会第 38 回大会、清水・静岡（2009）

45) 小島　肇、北條麻紀、新井晶子：3 次元培養表皮モデルを用いるコメットアッセイと細胞毒性の関係、日本環境変異原学会第 38 回大会、清水・静岡（2009）

46) JaCVAM:コメットアッセイ国際バリデーショ

ンプロジェクトチーム：インビボコメットアッセイ：JaCVAM 国際バリデーション試験の進捗状況報告、日本環境変異原学会第 38 回大会、清水・静岡 (2009)

47) 伊藤正俊、関東裕美、鷲崎久美子、松永佳世子、矢上晶子、中川真美子、加藤則人、河合恵一、滝脇弘嗣、吉村　功、小島　肇：パッチテストによる皮膚一次刺激性評価（2）、第 39 回日本皮膚アレルギー・接触皮膚炎学会総会学術大会、京都 (2009)

48) 中村昌文、半田洋士、小野 敦、小島 肇：Lumi-cell ER アッセイ法の国際バリデーション(第二報)、第 12 回環境ホルモン学会研究発表会、東京 (2009)

49) 小島　肇、飯島正文、松永佳世子、佐々　斉、板垣　宏、岡本裕子、西山直宏、小野寺博志、見田　活、鷲田　淳、益山光一、増田光輝、大野泰雄：あり方検討会設立の経緯及び動物実験代替法の現状、医薬部外品の製造販売承認申請における安全性に関わる資料のあり方検討委員会報告、東京 (2009)

50) 山本直樹、平野耕治、谷川篤宏、加藤雅一、畠賢一郎、小島　肇、綾木雅彦、堀口正之、谷口孝喜：角膜上皮細胞における組織幹細胞マーカーの検索と初代分離培養法および遺伝子導入法の検討、日本組織培養学会第 82 回大会、栃木 (2009)

51) 山本直樹、平野耕治、谷川篤宏、加藤雅一、畠賢一郎、小島　肇、綾木雅彦、堀口正之、谷口孝喜：角膜上皮細胞の組織幹細胞マーカーと初代分離培養法および遺伝子導入法の検討、第４１回日本臨床分子形態学会総会・学術集会、神戸 (2009)

52) 小島　肇：今後の展望、JaCVAM 第 3 回ワークショップ、h-CLAT シンポジウム、東京 (2010)

53) Kojima, H., Arai, S. and Hojyo, M.: Adequate conditions for performance of comet assay using 3-dimensional human epidermal model, 49th Annual SOT meeting, Salt Lake City (2010)

54) Stokes, W., Wind, M., Blakey, D., Kreysa, J., Kojima, H., Anklam E.: Establishment of the International Cooperation on Alternative Test Methods (ICATM) and Its Role in the Validation and Regulatory Acceptance of Globally Harmonized Safety Assessment Methods, 49th Annual SOT meeting, Salt Lake City (2010)

55) Ceger, P., Deal, F., Allen, D., Clark, G., Pazos, P., de Lange, J., Bremer, S., Nakamura, M., Kojima, H., Ono, A., Tice, R., Stokes, W.: Testing of Coded Substances in the NICEATM/ECVAM/JaCVAM LUMI-CELL® STTA Multiphase International Validation Study, 49th Annual SOT meeting, Salt Lake City (2010)

J. 添付資料

添付資料 1：Phase 4-1 Result
添付資料 2：Minutes, International meeting in Italy
添付資料 3：Minutes, International meeting in USA
添付資料 4：第 9 回国内委員会議事録
添付資料 5：第 10 回国内委員会議事録
添付資料 6：第 11 回国内委員会議事録
添付資料 7：Protocol International Validation of the in vivo Rodent Alkaline Comet Assay for the Detection of Genotoxic Carcinogens (Version 14.2)
添付資料 8：Study plan
添付資料 9：コメット画像例

Phase 4-1 Result

2010/02/04

T. Omori & M. Suzuki

Background

- Phase IV step 1 data.
- This study follows the protocol **version 14** and the study plan for it.
- The purpose of the study was to examine the extent of **reproducibility** and variability of assay results among laboratories using coded test chemicals and the positive control EMS.

2

Statistical methods

- Judgment was based on **Dunnett's test (two-sided, P<0.05)** for mean values of % tail DNA, according with the protocol.
- For the secondary interest, the result of trend test was also obtained.
- For assay sensitivity, Student's t test (one-sided, P<0.025) is conducted for mean values of % tail DNA.

3

What this slide show...

- **Graphical displays** of the results to related to the above mentioned statistical methods
  - Histogram of % tail DNA
  - Vehicle vs. Positive control
  - Vehicle, Low dose, Middle dose, and High dose
  - Effect with simultaneous confidence interval
  - Liner regression line

4

Chemical code

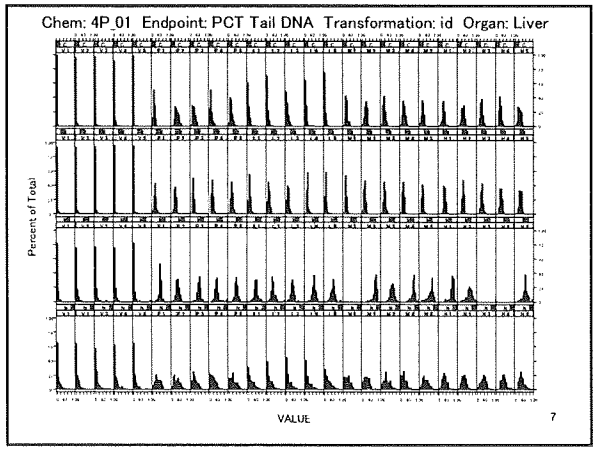
- Chem_01: EMS
- Chem_02: D-Mannitol
- Chem_03: 2_AAF
- Chem_04: MNU

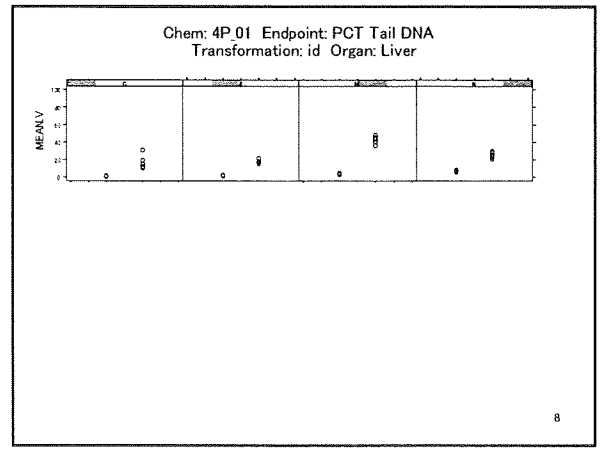
5

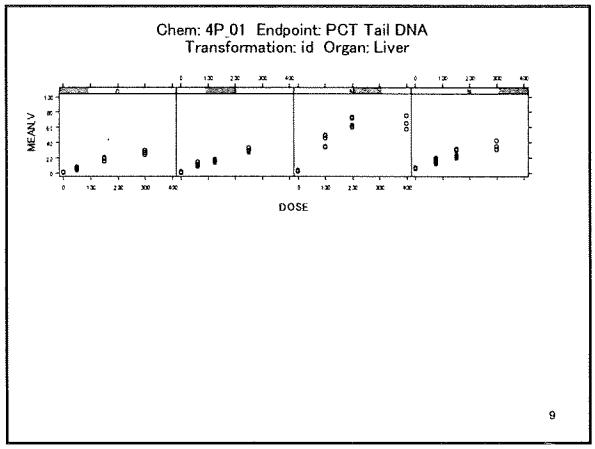
Laboratory code

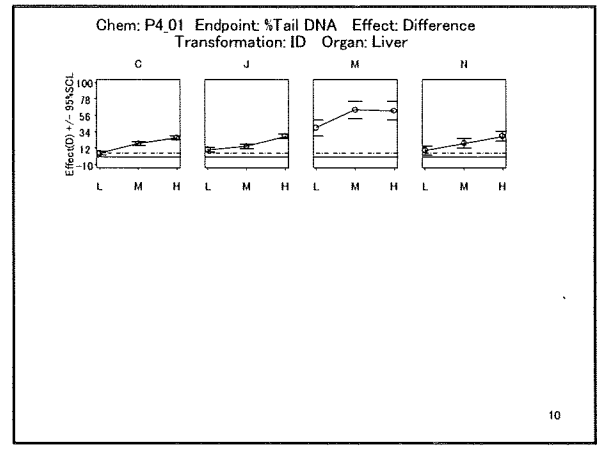
- Lead laboratory: B ~ E
- Other : F~N

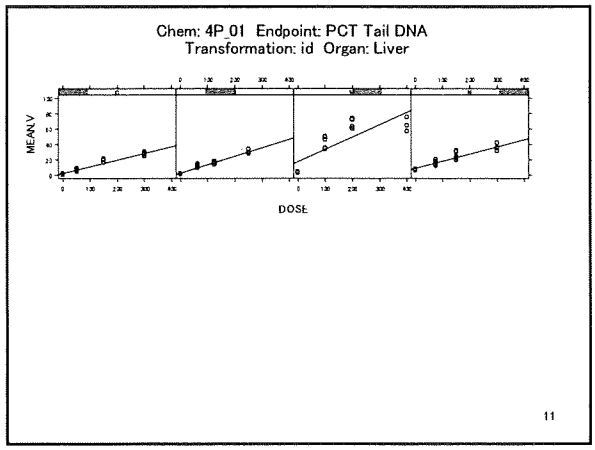
6

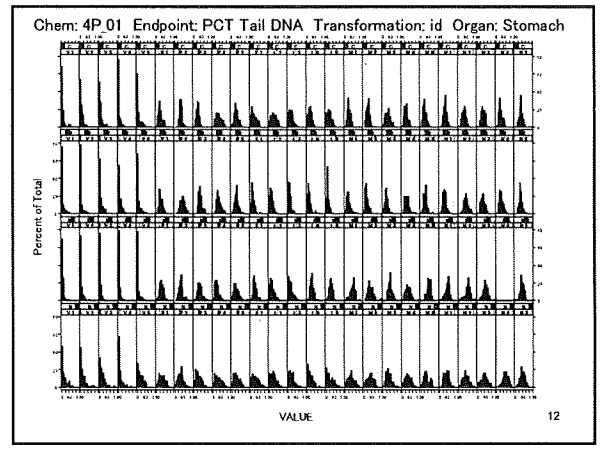

Chem: 4P_01 Endpoint: PCT Tail DNA Transformation: id Organ: Liver
Percent of Total
VALUE
7

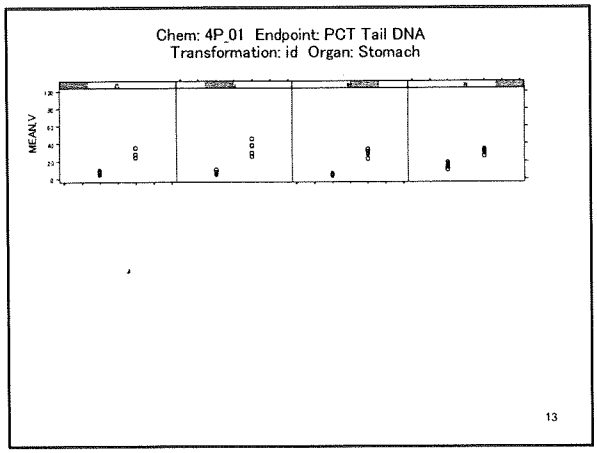

Chem: 4P_01 Endpoint: PCT Tail DNA
Transformation: id Organ: Liver
MEAN.V
8

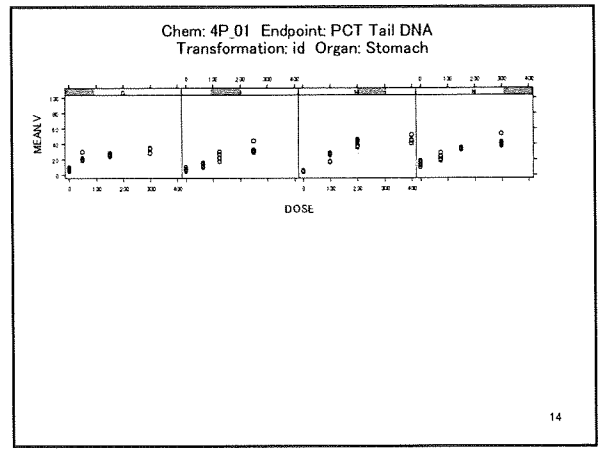

Chem: 4P_01 Endpoint: PCT Tail DNA
Transformation: id Organ: Liver
MEAN.V
DOSE
9

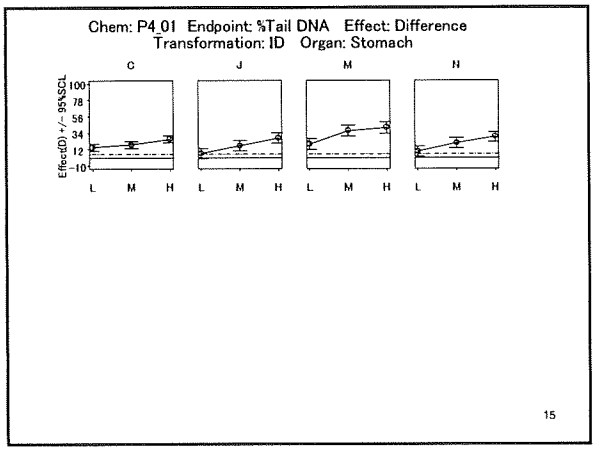

Chem: P4_01 Endpoint: %Tail DNA Effect: Difference
Transformation: ID Organ: Liver
C
J
M
N
Effect(D) +/- 95%SCL
100
78
56
34
12
-10
L
M
H
10

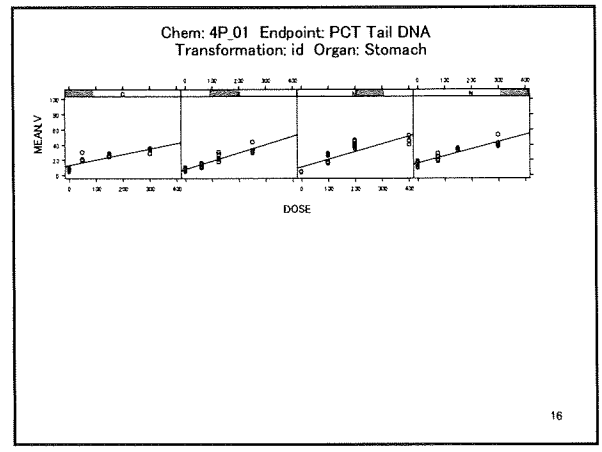

Chem: 4P_01 Endpoint: PCT Tail DNA
Transformation: id Organ: Liver
MEAN.V
DOSE
11

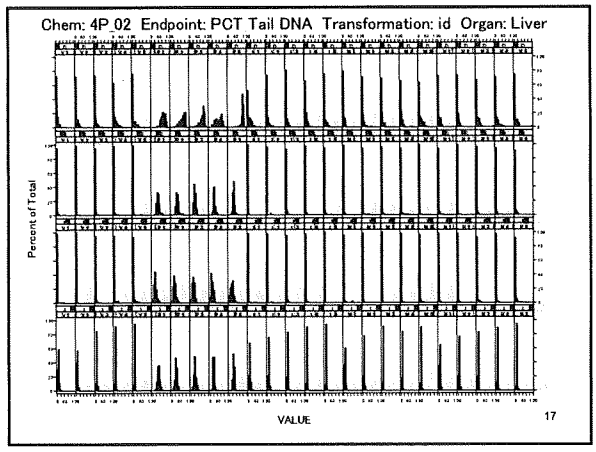

Chem: 4P_01 Endpoint: PCT Tail DNA Transformation: id Organ: Stomach
Percent of Total
VALUE
12

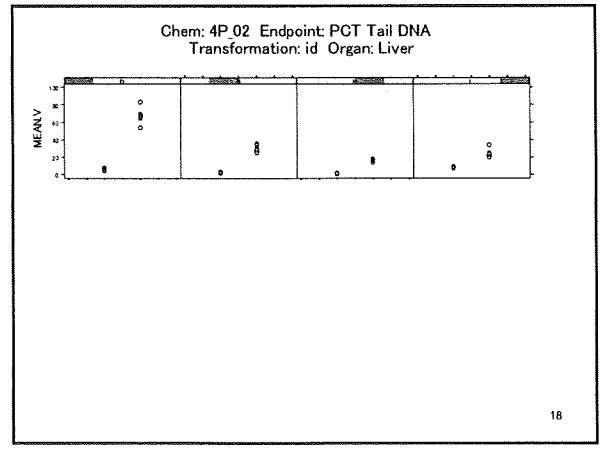

Chem: 4P_01 Endpoint: PCT Tail DNA
Transformation: id Organ: Stomach
MEAN.V
13

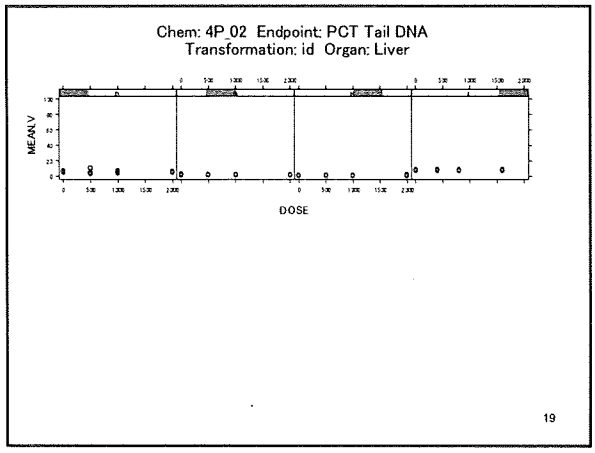

Chem: 4P_01 Endpoint: PCT Tail DNA
Transformation: id Organ: Stomach
MEAN.V
DOSE
14

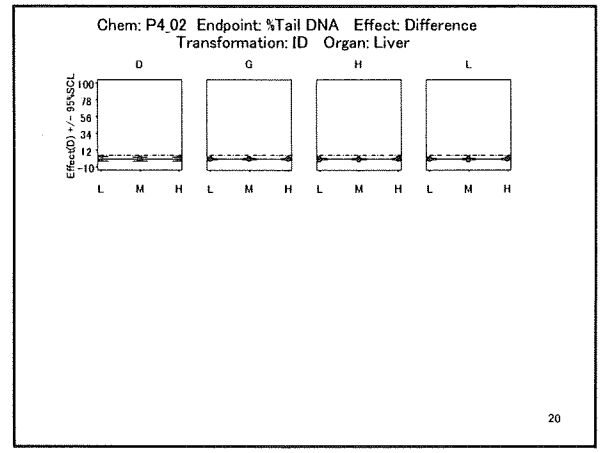

Chem: P4_01 Endpoint: %Tail DNA Effect: Difference
Transformation: ID Organ: Stomach
C
J
M
N
Effect(D) +/- 95%SCL
100
78
56
34
12
-10
L M H L M H L M H L M H
15

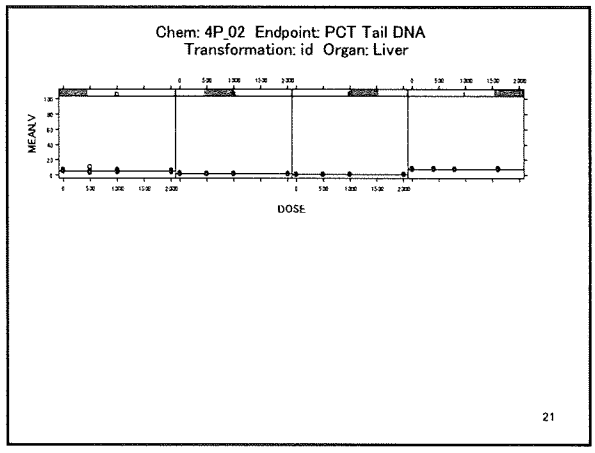

Chem: 4P_01 Endpoint: PCT Tail DNA
Transformation: id Organ: Stomach
MEAN.V
DOSE
16

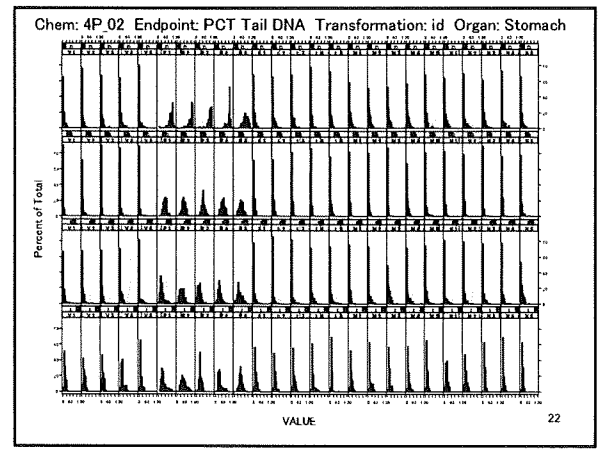

Chem: 4P_02 Endpoint: PCT Tail DNA Transformation: id Organ: Liver
Percent of Total
VALUE
17

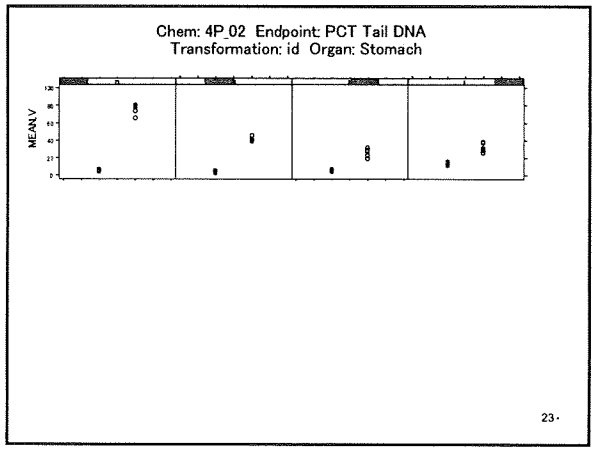

Chem: 4P_02 Endpoint: PCT Tail DNA
Transformation: id Organ: Liver
MEAN.V
18

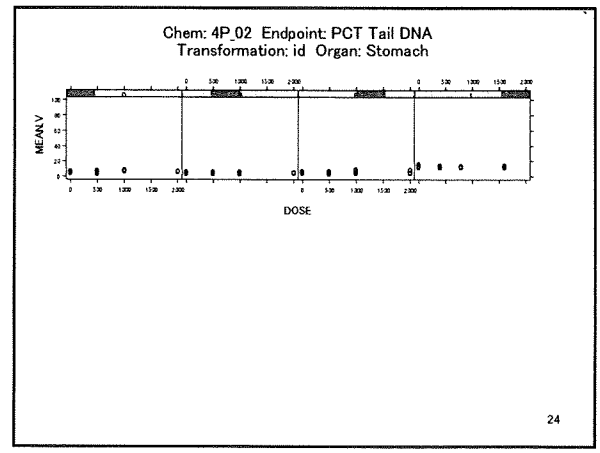

Chem: 4P_02 Endpoint: PCT Tail DNA
Transformation: id Organ: Liver
MEAN V
DOSE
19

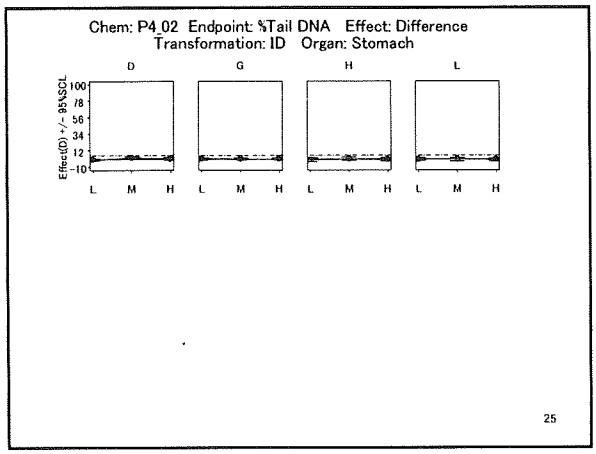

Chem: P4_02 Endpoint: %Tail DNA Effect: Difference
Transformation: ID Organ: Liver
D
G
H
L
Effect(D) +/- 95%SCL
100
78
56
34
12
-10
L M H L M H L M H L M H
20

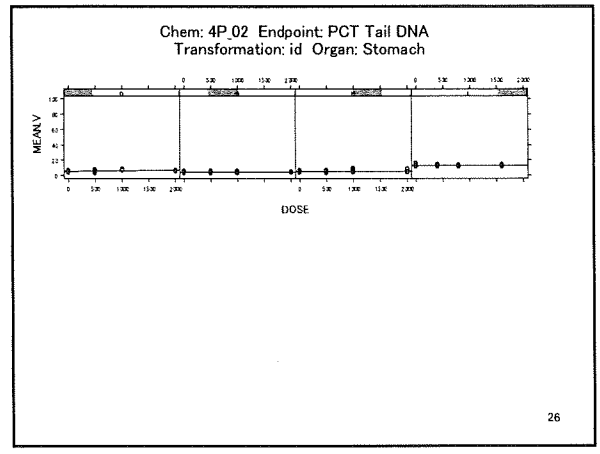

Chem: 4P_02 Endpoint: PCT Tail DNA
Transformation: id Organ: Liver
MEAN V
DOSE
21

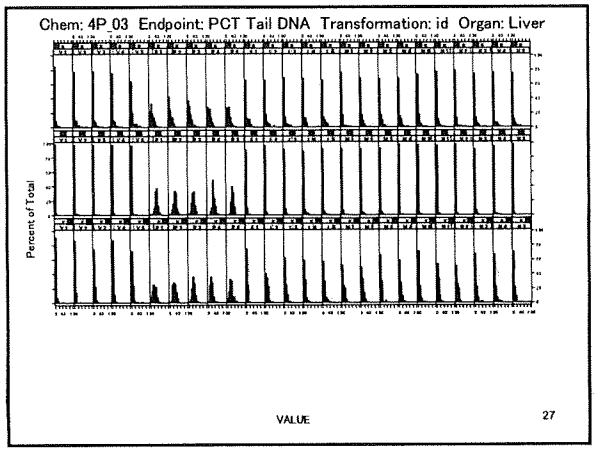

Chem: 4P_02 Endpoint: PCT Tail DNA Transformation: id Organ: Stomach
Percent of Total
VALUE
22

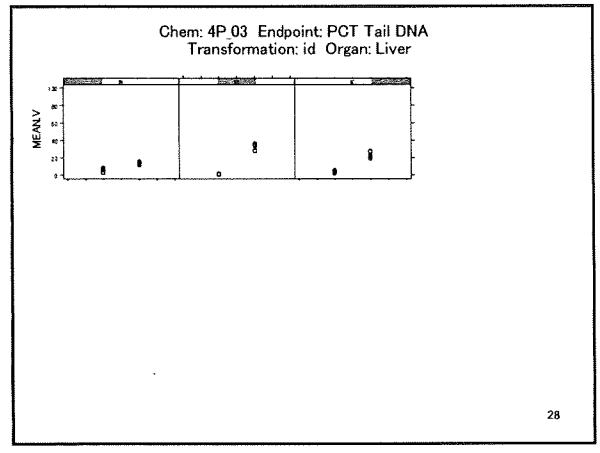

Chem: 4P_02 Endpoint: PCT Tail DNA
Transformation: id Organ: Stomach
MEAN V
23

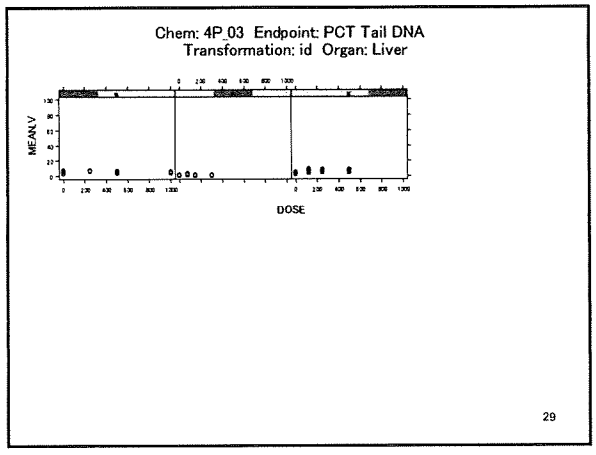

Chem: 4P_02 Endpoint: PCT Tail DNA
Transformation: id Organ: Stomach
MEAN V
DOSE
24

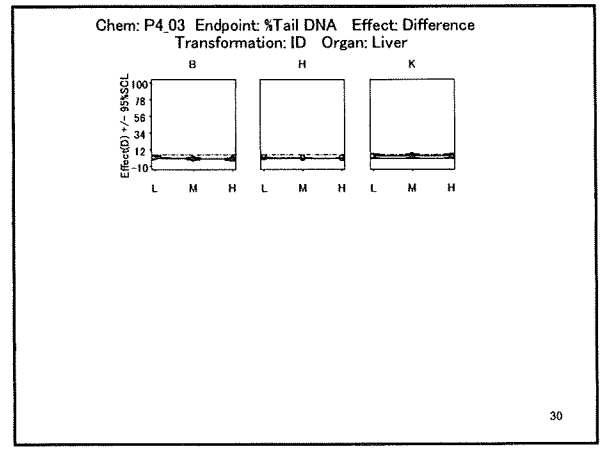

Chem: P4_02 Endpoint: %Tail DNA Effect: Difference
Transformation: ID Organ: Stomach
D
G
H
L
Effect(D) +/- 95%SCL
100
78
56
34
12
-10
L M H L M H L M H L M H
25


Chem: 4P_02 Endpoint: PCT Tail DNA
Transformation: id Organ: Stomach
MEAN.V
DOSE
26

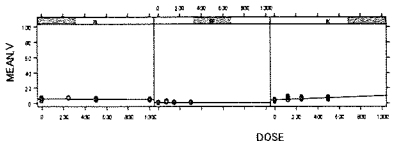

Chem: 4P_03 Endpoint: PCT Tail DNA Transformation: id Organ: Liver
Percent of Total
VALUE
27


Chem: 4P_03 Endpoint: PCT Tail DNA
Transformation: id Organ: Liver
MEAN.V
28


Chem: 4P_03 Endpoint: PCT Tail DNA
Transformation: id Organ: Liver
MEAN.V
DOSE
29

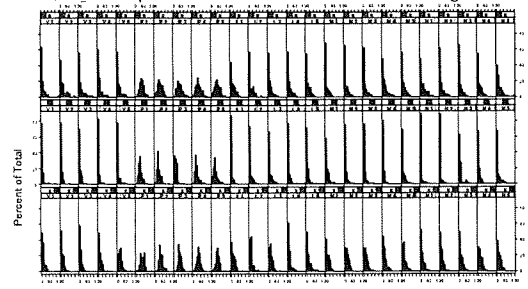

Chem: P4_03 Endpoint: %Tail DNA Effect: Difference
Transformation: ID Organ: Liver
B
H
K
Effect(D) +/- 95%SCL
100
78
56
34
12
-10
L M H L M H L M H
30

Chem: 4P_03 Endpoint: PCT Tail DNA
Transformation: id Organ: Liver



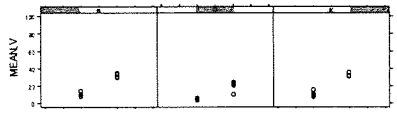
31

Chem: 4P_03 Endpoint: PCT Tail DNA Transformation: id Organ: Stomach



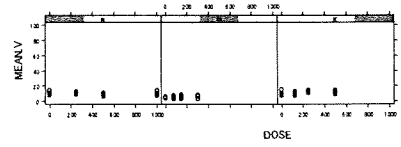
32

Chem: 4P_03 Endpoint: PCT Tail DNA
Transformation: id Organ: Stomach



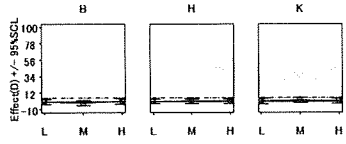
33

Chem: 4P_03 Endpoint: PCT Tail DNA
Transformation: id Organ: Stomach



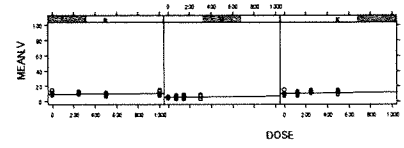
34

Chem: P4_03 Endpoint: %Tail DNA Effect: Difference
Transformation: ID Organ: Stomach

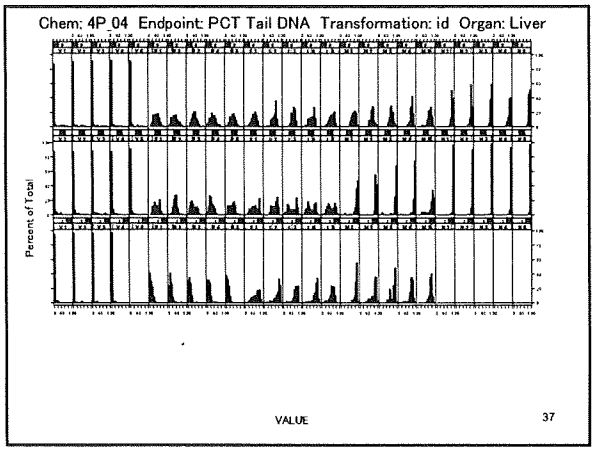


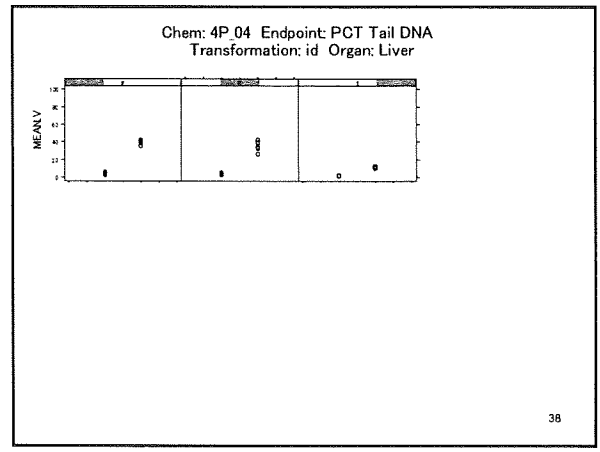
35

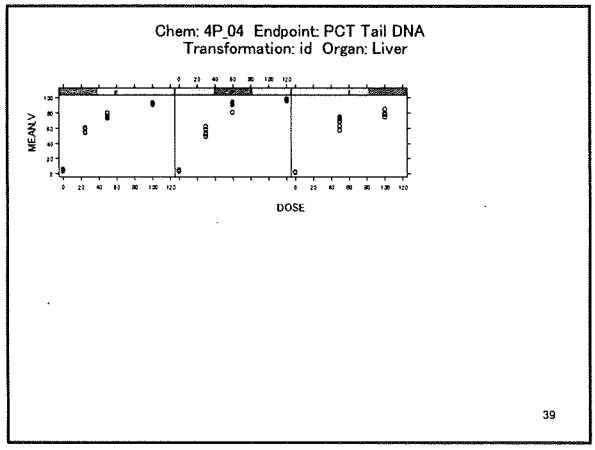
Chem: 4P_03 Endpoint: PCT Tail DNA
Transformation: id Organ: Stomach

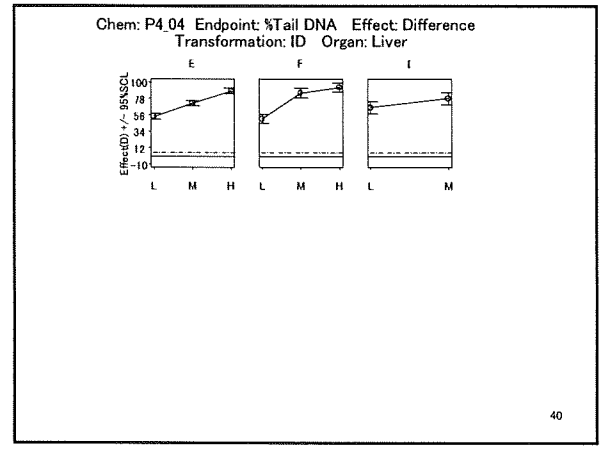


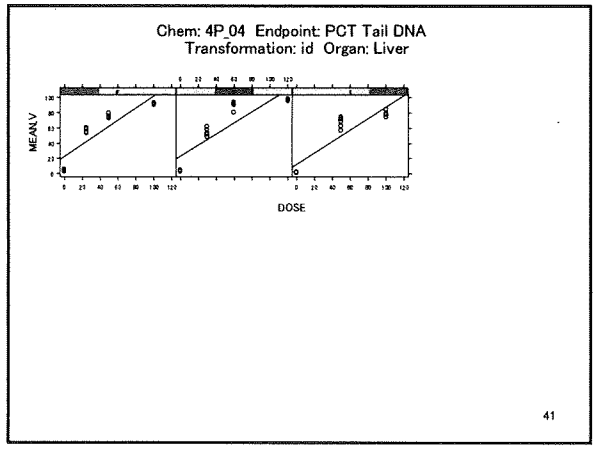
36

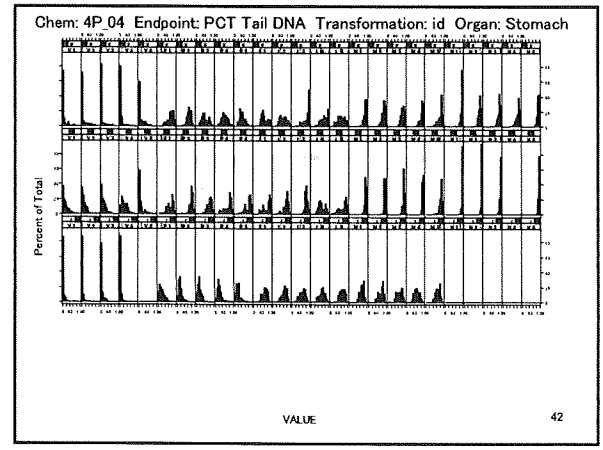

Chem: 4P_04 Endpoint: PCT Tail DNA Transformation: id Organ: Liver
Percent of Total
VALUE
37


Chem: 4P_04 Endpoint: PCT Tail DNA
Transformation: id Organ: Liver
MEANLV
38

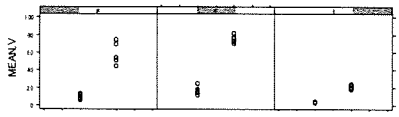

Chem: 4P_04 Endpoint: PCT Tail DNA
Transformation: id Organ: Liver
MEANLV
DOSE
39


Chem: P4_04 Endpoint: %Tail DNA Effect: Difference
Transformation: ID Organ: Liver
E
F
I
Effect(D)+/- 95%SCL
100
78
56
34
12
-10
L
M
H
L
M
H
L
M
40


Chem: 4P_04 Endpoint: PCT Tail DNA
Transformation: id Organ: Liver
MEANLV
DOSE
41

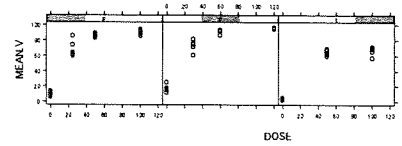

Chem: 4P_04 Endpoint: PCT Tail DNA Transformation: id Organ: Stomach
Percent of Total
VALUE
42

Chem: 4P_04 Endpoint: PCT Tail DNA
Transformation: id Organ: Stomach



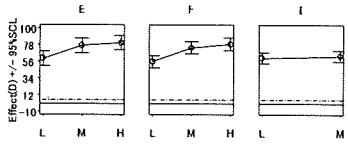
43

Chem: 4P_04 Endpoint: PCT Tail DNA
Transformation: id Organ: Stomach



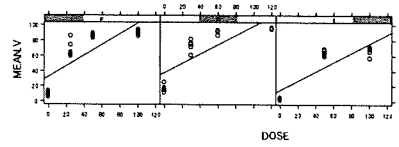
44

Chem: P4_04 Endpoint: %Tail DNA Effect: Difference
Transformation: ID Organ: Stomach



45

Chem: 4P_04 Endpoint: PCT Tail DNA
Transformation: id Organ: Stomach



46

Discussion 1

- These graphs showed evidence of the high **reliability** of this assay, for 4 chemicals, EMS, D-Mannitol, 2-AAF, and MUN.
- We don't still have enough data for evaluation of **relevance**.
- In the following steps, the results from many types of chemical would be needed.

47

Discussion 2

- Dunnett's test detected the very small effect in some dataset, but graphs suggested these effects may not be biologically significant.
- Incorporating the trend test with Dunnett's test for judgment using statistical test would be reasonable, in further steps.

48

Draft Minutes

The 6th meeting for the International Comet assay validation study

Date: August 25, 2009, 14:00 p.m.-August 26, 17:00 p.m.

Venue: Grand Hotel Baglioni, in Firenze, Italy.

August 25

Welcome address

1. On behalf of Dr. Makoto Hayashi (An-pyo Center: Chair of validation management team: VMT), Dr. Yoshifumi Uno (Mitsubishi Tanabe Pharma Corporation) as the acting co-chairs opened the meeting and welcomed participants. He introduced the topic and gave a presentation on the final goal of this project and the objective of the 6th meeting for the International Comet assay validation study.
2. Participants introduced themselves (for a list of participants see Annex 2).
3. Dr. Hajime Kojima (JaCVAM, NIHS) provided general information regarding the meeting.

In vitro Comet-international validation study

Dr. Masamitsu Honma (NIHS), as the acting co-chair, summarized the validation study results of phases 1 and 2. Following this, BioReliance Corporation (Dr. Jing Shi), Huntingdon Life Sciences (Dr. Brian Burlinson), Boehringer-Ingelheim Pharmaceuticals, Inc. (Dr. Patricia Escober), and the Food and Drug Safety Center Hatano Research Institute (Dr. Yuzuki Nakagawa) presented the results of each study in detail. Throughout the discussion, two major issues were identified: how to evaluate cytotoxicity and how to detect mutagens that require metabolic activation.

In the phase-2 validation in vitro study, each lab freely selected the treatment concentrations for each test chemical based on one of three endpoints of cytotoxicity (i.e., 80% TBDE [Trypan blue dye exclusion test], 20% NDCN [counting non-detectable cell nuclei; hedgehog], or no cell growth for 24 hr in RSG [relative cell growth]). Overall, the lowest maximum concentration was selected when RSG was used as the cytotoxic parameter, and thus the reason why the negative results were obtained with the mutagens expected to be positive with/without S9 mix was likely due to the selection of lower concentrations rather than the issue of metabolic activation. In conclusion, a test chemical should be treated in an in vitro comet assay up to the solubility limit with the most appropriate solvent, and three or more,,cytotoxicity parameters (e.g., ATP assay) would be collected until sufficient data become available to determine the most suitable cytotoxicity parameter. In addition, it is unnecessary to change the metabolic activation conditions used in this assay (e.g.. S9 concentration), because the same conditions are used for in vitro mutational assays with this cell line.

The submission for publication of study results is now pending, because some practical aspects that should be optimized in the study protocol are still remaining.

August 26

In vivo data analysis and next study plan

Interim data on the 1st step of the 4th phase of the validation study were reported from Bayer Schering Pharma (Dr. Uta Wirnitzer), MCM: Mitsubishi Chemical Medience Corporation (Dr. Hironao Takasawa), FDSC (Dr. Yuzuki Nakagawa), IET: The Institute of Environmental Toxicology (Dr. Kunio Wada) and Merck (Dr. Uno for Dr. Andrew Kraynac). Dr. Uno summarized results of the lab recruitment phase and the 3rd phase of the validation study. Participants felt that the results of the lab recruitment phase should not be published until some issues are resolved (i.e., discordant data were obtained, especially for acrylamide but the difference in results might be related to dose selection).

There was discussion about the study protocol v.14. Some participants suggested that 'at least 20 min' was needed as electrophoresis duration, and this was accepted. The most important point in electrophoresis was that the negative control values be in the target ranges (i.e., 1-8% in the liver and 1-20% in the stomach) by controlling the electrophoresis duration suitably. The following were discussion points raised by participants and the VMT will discuss and decide what action is appropriate for these issues later: should the VMT specify the vehicle and dose levels for coded test chemicals?; should the administration regimen be modified to enable detection of MN in peripheral blood (e.g., 4 days)?; should the duration of tissue sampling be kept to a set time (e.g., within 10 min) in order to obtain more stable negative control values?; should the duration for lysis be controlled to obtain more stable negative control values?; should the response for the positive control group be based on a statistical analysis only or would data be acceptable when the positive control showed a clear positive response?; and should there be careful consideration about statistics when one negative control group is used for two separate chemical responses?

Dr. Uno presented two topics (i.e., possibility of reduction of animal numbers in the positive control group, and comparison of statistical analysis results between 2 slides and 3 slides). The following were comments from participants: animal reduction was preferred but the size of the positive control group should be always be appropriate for the in vivo comet assay; 80% probability for a positive response would be too low, and halfway reduction of animals might result in negative judgment in the positive control group followed by an additional experiment with more animals[LMS1]; and 1 slide might be sufficient to be analyzed (this possibility should be evaluated upon completion of the validation study).

Dr. Uno presented a statistics discussion. In this validation study, Dunnett and linear trend tests would be used for data analysis using the individual animal as the source for the error term.

In vivo Comet-international validation study

Workshop - Image Analysis with Comet IV

Dr. Madoka Nakajima (An-pyo Center) presented judgment criteria for comet image analysis by using a color atlas. Throughout the discussion about image analysis, participants reached a consensus that cells should be classified into three categories, scorable, non-scorable, and hedgehog, and also that scorable cells with a 90% or more DNA in the tail should be excluded from data analysis. Dr. Nakajima will send the comet photos without numerical data to labs by the middle of September, each lab will classify the comet photos into the three categories by the end of September, and then the color atlas will be revised and distributed to labs by the end of October. (Note: the categories will be decided by a majority vote.)

Adjourn

Draft Minutes

The 7th meeting for the International Comet assay validation study

Date: March 12, 2010, 9:00 a.m - 5:00 p.m.

Venue: Hilton Hotel, Salt lake City, USA.

Welcome address

1. Dr. Makoto Hayashi (BSRC: Chair of validation management team: VMT) opened the meeting and welcomed participants.
2. Participants introduced themselves (for a list of participants see Annex 2).
3. Dr. Hajime Kojima (JaCVAM, NIHS) provided general information regarding the meeting.

Catalogue of Comet Image

Dr. Makoto Hayashi presented judgment criteria for comet image analysis by using a color atlas prepared by BSRC/MMS. It was explained that the classification was mainly depend on the voting by the experts after the Florence meeting. There were still several comments and objections. The consensus at the morning meeting was it is important and helpful for the evaluation of the comet assay. Also, several volunteers would try to make re-evaluation each image for consensus call.

After the main meeting, small separate group met and tried to make consensus call for images one by one. Based upon the suggestions and also comments and suggestions sent by Dr. Tice after the meeting, BSRC will work on finalizing the atlas.

In vitro Comet-international validation study

[1] Summary of the Phase II pre-validation study

Dr. Masamitsu Honma (NIHS) as the acting co-chair, summarized the Phase II pre-validation study. He took same presentation in the 6th meeting in Firenze. In the Firenze meeting, some issues were considered as causes of inter-laboratory difference. After the Firenze meeting, he surveyed experimental conditions in each laboratory to figure out the causes of inter-laboratory difference. Critical points are as follows;

1. Treatment or culture condition
   a) Volumes during treatment and culture
   b) Plastic ware for the treatment and culture (tube, well, or flask)
   c) In the original protocol, 10ml for the treatment in 15ml tube and 10ml for culture in TS25 flask

      Conclusion: It is important to unify the treatment and culture condition. The treatment with enough volume my avoid loss of cell during preparation.
2. Solvent for chemical treatment
   a) Saline, Water, DMSO

      Conclusion: The choice of solvent did not influence the Comet results of at least 6 chemicals in the

Phase II study. However, an appropriate way to choose the solvent is needed.

3. Cytotoxicity criteria for dose-finding

a) No cell growth by RSG

b) 80% of TBDE

c) 20% NCDN

Conclusion: They are not equivalent. There is no appropriate cytotoxicity test to find the top concentration in the in vitro Comet assay currently. In vitro Comet assay should be performed under wide dose range (0.1-3000 ug/ml) regardless cytotoxicity. A new cytotoxicity parameter (e.g. CellTiter-Glo for ATP) could be tried.

4. S9

a) In vitro Comet assay dose not work well for chemicals requiring metabolic activation under S9, although other genotoxicity tests (MN) appropriately work in the same condition.

Conclusion: It is a property of in vitro Comet assay.

In conclusion, the phase II pre-validation study was appropriately conducted. Genotoxic chemicals not requiring metabolic activation (9AA, CAM, ETO) were judged as positive by almost laboratories, and a non-genotoxic chemical (MAM) was judged as negative by all laboratories. Two chemicals requiring metabolic activation (CP, DMN) exhibited very weak positive response with metabolic condition. Overall, in vitro Comet assay can be used for detecting genotoxic hazards. However, inter-laboratory difference may generate in the current protocol. In particular, the different dose-setting from dose-finding cytotoxicity test is critical to cause the inter-laboratory difference. He recommended that the in vitro Comet assay should be performed under wide dose range (0.1-5000 ug/ml) regardless cytotoxicity. The phase III pre-validation study will verify the new protocol.

[II] Plan of the Phase III validation study

Dr. Masamitsu Honma (NIHS) proposed the Phase III pre-validation study, and introduce its plan.

i) Organization (Leading laboratories)

In addition to original 4 laboratories, one Korean laboratory will join the study.

1. Bio-Reliance, USA (K. Pant)
2. Boehringer-Ingelheim, USA (P. Escobar)
3. Food and Drug Safety Center, Japan (K. Yamakage)
4. Huntington Life Sciences, UK (B. Burlinson)
5. Korean Laboratory

The Korean laboratory will newly join the Phase III validation study. To confirm their skill of the in vitro Comet assay, they must examine two chemicals, which were used in Phase I or II trial, according to Phase III protocol. After evaluating the results by Japanese and Korean VMT members, they will be allowed to join the validation study.

ii) Test chemicals

VMT selects 2 chemical. They will be 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. The method for selecting solvent will be addressed in detail. Ray gave information of the method.

iii) Protocol

The test will be conducted under same doses regardless cytotoxicity. Cytotoxicity will be measured, and it will be retrospectively used considering the weight of evidence.

1. Experimental doses are provided (both with and without S9)

   0, 0.15, 0.5, 1.5, 5.0, 15, 50, 150, 500, 1500, 5000 ug/ml (11 doses) with and without S9

2. Positive Control both for with and without S9

   EMS; 500 ug/ml

3. Duplicate

4. Cytotoxic parameter

   Relative cell growth (RSG) for 24 h after the treatment

   Counting non-detectable cell nuclei (NDCN; hedgehog)

   CellTiter-Glo or other methods for ATP can be optionally used (It is measured just after treatment).

5. 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.(Post-Mitochondrial Supernatant: Sprague-Dawley rat liver, Male,Phenobarbital/5,6 Benzoflavone induced) must be commonly used in the study (cat.# 11-105).

iv) Action plan

2010.4 Protocol will be provided.

2010.6 Test chemicals will be delivered. The Validation study will start.

2010.11 Deadline for collecting data.

2011.2 VMT meeting for validating the Phase III study, and for planning the next step.

Korean laboratory will join the study after confirming the skill of the Comet assay

In vivo data analysis and next study plan

1. Dr. Uno presented data of the 4th phase-1st step validation study with a PP file ("4th phase-step 1_100312.ppt"). All participants accepted the conclusion and the discussion. Dr. Beevers informed participants of her experience that 2-AAF showed positive response in the liver, and suggested that positive detection of 2-AAF might be related to some experimental conditions such as the metabolic activation, dosing regimen, and sampling time.
2. Dr. Uno informed participants that a few Japanese laboratories asked VMT about acceptability of the negative control values below 1% in the liver. VMT decided not to accept such changes, because it would allow too sensitive judgment for positive responses of test chemicals. Participants in this meeting accepted the VMT decision. It was suggested that prolongation of electrophoresis duration and increase in alkali solution temperature within 10°C would be effective to increase the negative control levels above 1%.
3. Dr. Uno informed participants that a few Japanese laboratories asked VMT about acceptability to include cytotoxic findings in gross pathology (preferably histopathology) into the reasons for dose

selection. He explained the background of this proposal with a PP file ("example.ppt") prepared by Lab. I. Many participants felt that MNU would be positive in the stomach based on the data of 6.25 mg/kg group because no cytotoxicity was noted at the dose level. Comments on the Lab. I proposal were as follows: only gross pathology results would be insufficient to indicate cytotoxicity; histopathological examination using animals of dose-finding studies would be unfeasible due to much time and effort needed; fixed dose procedure in OECD guidelines would be useful to select suitable dose levels; and there is no critical issue when either liver or stomach shows positive results without cytotoxicity (MNU induced positive responses in the liver without cytotoxicity, and thus positive in the comet assay). Participants in this meeting felt that dose levels should be selected based on descriptions in the study protocol as it was. VMT asked participants about acceptability of additional comet assays with lower dose levels if all dose levels used in the first assay induce cytotoxicity for both liver and stomach. Participant laboratories in the meeting accepted the proposal. In addition, following were ideas suggested in the meeting: histopathological examination even when negative results were obtained in comet assay; and peer review by NIEHS pathologists to obtain equalized histopathological findings.

4. Judgment criteria were discussed. The study protocol mentions that positive or negative is judged with statistical analysis, but the positive call seems to be questionable when the magnitude of responses is within the negative control background ranges. VMT consider that the judgment should be done based on the statistical analysis results in accordance with the study protocol, but it will be discussed again after the data of validation study are available.

Adjourn

第９回コメットアッセイ国内委員会議事録

日　時：平成 21 年 4 月 17 日 (金) 10 時～12 時 20 分
場　所：国立医薬品食品衛生研究所　第一会議室
出席者：林　真、宇野芳文、本間正充、浅野哲秀、森田健、中嶋圓、鈴木雅也、山影康司、中川ゆづき、
田中憲穂、小島肇　　以上順不同、敬称略

議事：
小島委員が司会を務め、議事を進行させた。

１．前回議事録確認
資料１に示すように、２月の大阪国際会議の開催協力に林委員長および小島委員より感謝の言葉が述べられた。

２．２月以降の動向と今後の予定について
小島、宇野および本間委員より、２月以降の本研究に関する動向が説明された。

１）小島委員が２月中旬に資料６を用いて、コメットアッセイのバリデーション研究を含む研究班の中間報告を行った。評価委員からの進捗に関する質問は厳しいものがあったと説明された。

２）結果として、資料２の申請書に示すように研究費は大きく減らされたことから、本研究に関する予算も減らさざるを得ないとの方針を大野班長と相談の上で決めた。コメットアッセイの国際バリデーション研究については、MMS 研究会の委託費を減らす、in vitro 研究の開始を延期する提案が小島委員よりなされ、了承された。宇野 in vivo 実行委員長より、研究費の多くを占める被験物質の発送経費を施設負担にする提案がなされ、小島委員が各施設への打診を約束した。

３）PhaseⅣ-1 については、5 月開始、次回実行委員会までのデータ収集の提案が小島委員よりなされた。本案も了承され、４月 20 日以降に被験物質を各施設に配布するとになった。宇野 in vivo 実行委員長より、実験の開始連絡が４月中に、プロトコールおよび判定アトラス（中嶋氏の担当）の配布が５月中旬になされる。

小島委員からの要請事項として、次回の国際バリデーション実行委員会（以後、国際実行委員会と記す）までに、in vivo では①PhaseⅢおよび PhaseⅣバリデーション研究（以降、バリデーション研究を省略）のための施設選抜試験の結果をまとめる、②PhaseⅣ-2 のための被験物質を固める、③データを固め、全体的なストーリーを作る。in vitro では④PhaseⅡまでの結果のまとめが挙げられた。また、年末までに、⑤これまでの結果を報告書にまとめる（分担、まとめ方は次回の国際実行委員会までに決定）、⑥PhaseⅣ-1 の結果をまとめることが要請された。

４）小島委員より、次回の国際実行委員会の議題として、in vivo の結果報告、プロトコールおよび PhaseⅣ計画の説明、判定アトラスの説明（判定基準の徹底：中嶋委員の担当）、in vitro 結果の報告とプロトコールの改正が挙げられた。PhaseⅣ-1 実施時における判定基準に対する疑問については、安評センターが対応することになった。

５）宇野委員より、集まったデータのチェックシステムおよびデータ整備について大森委員、北條氏（国立衛研）と共同で検討している。資料 9-11 の議事録に示すように、これまでメール、電話会議を含め３回の会議を開催したと報告された。大森委員がデータセットおよびデータチェックプログラムを SAS で作って実行し、確認したと説明された。今後、データの整理を北條氏にお願いしたいと依頼されたが、小島委員が室内業務との調整が済んでいないことから承諾せず、結論は保留となった。林委員長より、今後、解析するデータ数を減らすべく指標を絞り込むよう要望が示された。このグループ会議は方式を変更せず継続し、今後、林委員長および鈴木委員も参加することになった。

一方、資料８に示すように、陽性対照群の動物数の削減についてまとめたと宇野委員より報告された。国内委員会の結論として、解析内容に異論はないが、PhaseⅣのプロトコールには反映させないことになった。

６）本間委員より、in vitro データについては鈴木委員が解析中であり、近日中にまとめると説明された。５月 25 日電話会議の開催が提案された。

７）対外発表については、小島委員より、８ 月末に開催さる 7th World Congress on Alternatives & Animal Use in the Life Sciences に２つの演題 (in vitro および in vivo) を投稿したと説明された (資料 3)。8 月の 10th International Conference on Environmental Mutagens (ICEM)や 11 月の日本環境変異原学会大会にも発表をお願いしたいと要望がなされた。本間委員より、要旨集には参加施設名も記載すべきとの提言があった。

3．次回の国際実行委員会について

資料4に示すように、次回の国際実行委員会を8月25日午後～26日終日、フィレンツエで開催することが提案され、決定された。小島委員が早急に実行委員および PhaseⅣ参加施設に会議開催について連絡することになった。

配布資料

1）Draft Minutes, The 5th meeting for the International Comet assay validation study

2）厚生労働科学研究補助金交付申請書

3）7th World Congress on Alternatives & Animal Use in the Life Sciences

4）Phase IV-1 and Next meeting

5）International Validation study of in vivo comet assay, Outline of 4th phase validation study

6）厚生労働科学研究補助金(化学物質リスク研究事業)研究成果の概要

7）総合研究報告　遺伝毒性試験法のバリデーション研究

8）Short Letter, How many animals in positive control group can we reduce? From the in vivo comet assay variation project

9）in vivo Comet Assay バリデーション打ち合わせ議事録

10）第2回 in vivo Comet Assay バリデーション打ち合わせ議事録

11）第3回 in vivo Comet Assay バリデーション打ち合わせ議事録

以上

第10回コメットアッセイ国内委員会議事録

日　時：平成21年7月30日(木)10時～12時30分
場　所：国立医薬品食品衛生研究所　センター議室
出席者：林　真、宇野芳文、本間正充、浅野哲秀、森田健、中嶋圓、大森崇、小島肇
　　以上順不同、敬称略

議事：

１．イタリア国際実行委員会の進行について

小島委員より、agenda(資料1)および参加者情報（資料3)、会場情報（資料4）をもとに概要が説明された。小島委員の提出したAgendaは了承されたが、現場で柔軟に時間変更することで合意された。

アトラスについては、事前に送付し質問を促すとともに、判断に悩む画像の提示や，プロトコールの問題点に関する提案を期待する情報を流し、有意義な意見交換を目指すとされた。Agendaとともにatlasの早急な送付が要望として示された。ただし、以後、プロトコールは改訂せず、要望についてはその理由を説明して受け付けないことが宇野in vivo委員長より明言された。

２．データ解析動向と今後の予定

2-1　in vivo

In vivo 3rd phase バリデーション研究、Lab選択試験の結果が宇野 in vivo 委員長より報告された。これらの結果をICEMやIWGTなどで報告すると説明された。3rd phaseまでの結果解析から、電気泳動条件の固定化により安定な結果が得られることが明らかになっており、4th-1　phaseは良い結果が予想されるとされた。判定基準としては、「陰性対照との差が５％以上で有意差あり」がもっとも良いと考えていると説明された。

大森委員が検討してきた陽性対照匹数の削減やスライド数の適正数などの検討も報告され、次回の実行委員会で了承さえ得られれば、プロトコールを確定できると説明された。林委員長よりの提案として、次の段階としてOECDガイドライン案を想定した文書化も必要とされた。なお、バリデーション研究では統計解析に関する議論はすべきでないとの見解が示された。

4th-2　phaseの被験物質に関しては、1物質を何施設で実験するかについて意見交換がなされた。宇野委員より、小島委員より1物質／3施設を求める意見も受けているが、より少ない施設での実施を提案していきたいとされた。ともかく、少なくとも4th　phaseで20物質の結果を集めたいとされた。被験物質の選択に関しては、小島委員からICCVAMの意見を再確認することになった。主な比較データは不定期DNA合成であることが確認された。4th-1　phaseでは予備試験にあたる急性毒性試験方法や被験物質の扱いが不徹底であったことから、次のphaseでは1物質当たりの配布量を25g以上とするとともに、参加者や化学物質管理者の登録書式を作成する提案が宇野委員より示された。

２）in vitro

本間 in vitro 委員長より、in vitro 2nd phaseの結果が報告された。細胞毒性の指標として、トリパンブルーの取り込み(80%)、RSG（24時間後の増殖の有無）およびヘッジホッグ(20%)を毒性基準として検討してきたが、RSGは役にたたないことが明らかになった。細胞毒性の解釈が難しく、トリパンブルーの取り込みやRSGを考慮せず、高用量から機械的に処理するべきとの意見もあった。ヘッジホッグの判定も施設により異なり、atlasを用いた再教育が必要との見解も本間in vitro委員長より示された。