Endpoint: %Tail DNA Effect: Difference
Transformation: ID Organ: Liver

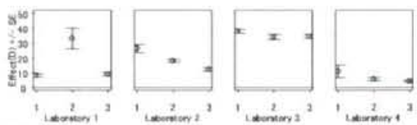

Fig. 9-L

Table 10-L

Organ: Liver Endpoint: PCT tail DNA
Transformation: id Link: id
Effect: Difference

			Lab											
			1			2			3			4		
Type	Repeat	Variable	Effect(D)	SE	Variance	Effect(D)	SE	Variance	Effect(D)	SE	Variance	Effect(D)	SE	Variance
INTRA				[illegible]	[illegible]		[illegible]	[illegible]		[illegible]	[illegible]		[illegible]	[illegible]
EXP	1	id(MEAN V)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	2	id(MEAN V)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	3	id(MEAN V)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Endpoint: %Tail DNA Effect: Ratio
Transformation: ID Organ: Liver

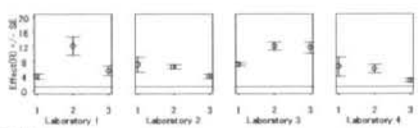

Fig. 10-L

Table 11-L

Organ: Liver Endpoint: PCT tail DNA
Transformation: id Link: id
Effect: Ratio

			Lab											
			1			2			3			4		
Type	Repeat	Variable	[illegible]	SE	Variance	[illegible]	SE	Variance	[illegible]	SE	Variance	[illegible]	SE	Variance
INTRA				[illegible]	[illegible]		[illegible]	[illegible]		[illegible]	[illegible]		[illegible]	[illegible]
EXP	1	id(MEAN V)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	2	id(MEAN V)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	3	id(MEAN V)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Table 12-L

Organ: Liver Endpoint: PCT tail DNA
Transformation: id Link: id
Effect: Difference

Variable	Component		SE	Variance
id(MEAN V)	Lab.	–	12.19	148.67
	Repeat(Lab.)	–	5.23	27.35
	Residual	1-1	1.11	1.23
		1-2	7.07	50
		1-3	1.37	1.88
		2-1	2.65	7.05
		2-2	0.91	0.84
		2-3	1.08	1.16
		3-1	1.13	1.28
		3-2	1.89	3.57
		3-3	1.36	1.85
		4-1	4.25	18.03
		4-2	1.05	1.1
		4-3	0.48	0.23

Table 13-L

Organ: Liver Endpoint: PCT tail DNA
Transformation: id Link: id
Effect: Ratio

Variable	Component		SE	Variance
id(MEAN V)	Lab.	–	1.83	3.34
	Repeat(Lab.)	–	2.43	5.89
	Residual	1-1	0.74	0.55
		1-2	2.52	6.34
		1-3	1.31	1.71
		2-1	2.1	4.42
		2-2	0.58	0.34
		2-3	0.59	0.34
		3-1	0.39	0.16
		3-2	1.08	1.18
		3-3	1.51	2.29
		4-1	2.58	6.68
		4-2	1.24	1.54
		4-3	0.38	0.15

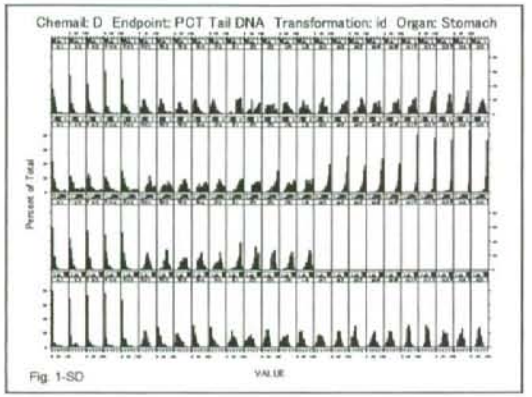

Fig. 1-SD

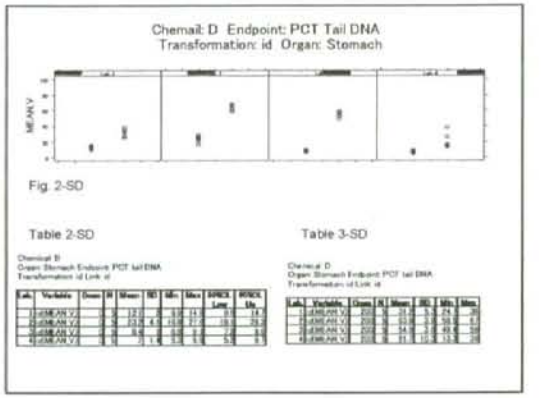

Fig. 2-SD

Table 2-SD

Table 3-SD

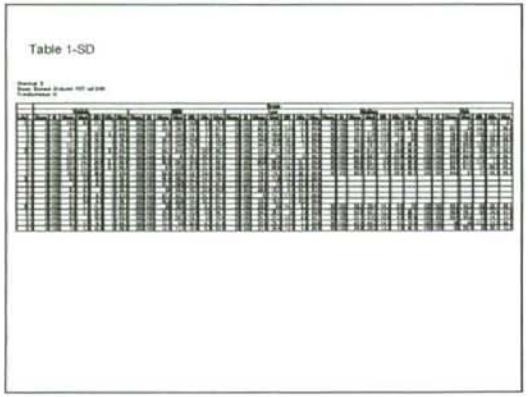

Table 1-SD

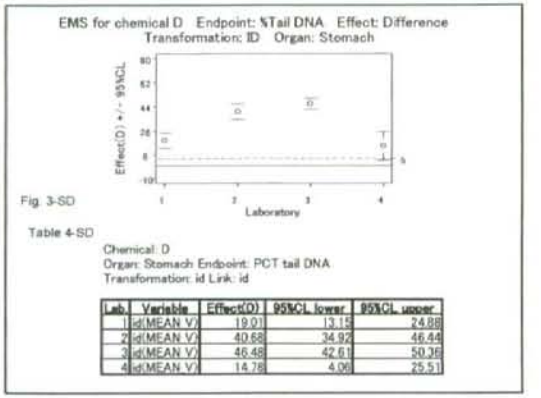

Fig. 3-SD

Table 4-SD

Chemical: D
Organ: Stomach Endpoint: PCT tail DNA
Transformation: id Link: id

Lab.	Variable	Effect(D)	95%CL lower	95%CL upper
1	id(MEAN V)	19.01	13.15	24.88
2	id(MEAN V)	40.68	34.92	46.44
3	id(MEAN V)	46.48	42.61	50.36
4	id(MEAN V)	14.78	4.06	25.51

EMS for chemical D Endpoint: %Tail DNA Effect: Ratio
Transformation: ID Organ: Stomach

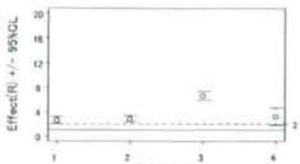

Fig. 4-SD

Table 5-SD

Chemical: D
Organ: Stomach Endpoint: PCT tail DNA
Transformation: id Link: id

Lab.	Variable	Effect(R)	95%CL lower	95%CL upper
1	id(MEAN V)	2.57	2.08	3.17
2	id(MEAN V)	2.75	2.36	3.28
3	id(MEAN V)	6.51	5.81	7.34
4	id(MEAN V)	3.12	1.79	4.67

Chemical: D Endpoint: %Tail DNA Effect: Difference
Transformation: ID Organ: Stomach

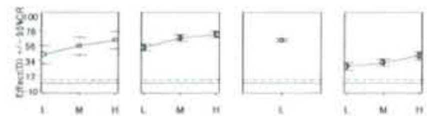

Fig. 6-SD

Table 7-SD

Chemical: D
Organ: Stomach Endpoint: PCT tail DNA
Transformation: id Link: id

		V - L			V - M			V - H		
Lab.	Variable	Effect(D)	95%CR lower	95%CR upper	Effect(D)	95%CR lower	95%CR upper	Effect(D)	95%CR lower	95%CR upper
1	id(MEAN V)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
2	id(MEAN V)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
3	id(MEAN V)	[illegible]	[illegible]	[illegible]						
4	id(MEAN V)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Chemail: D Endpoint: PCT Tail DNA
Transformation: id Organ: Stomach

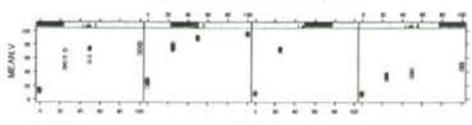

Fig. 5-SD

Table 6-SD

Chemical: D
Organ: Stomach Endpoint: PCT tail DNA
Transformation: id Link: id

Lab.	Variable	Dose
1	id(MEAN V)	[illegible]
2	id(MEAN V)	[illegible]
3	id(MEAN V)	[illegible]
4	id(MEAN V)	[illegible]

Chemical: D Endpoint: %Tail DNA Effect: Ratio
Transformation: ID Organ: Stomach

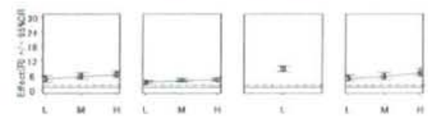

Fig. 7-SD

Table 8-SD

Chemical: D
Organ: Stomach Endpoint: PCT tail DNA
Transformation: id Link: id

		V - L			V - M			V - H		
Lab.	Variable	Effect(R)	95%CR lower	95%CR upper	Effect(R)	95%CR lower	95%CR upper	Effect(R)	95%CR lower	95%CR upper
1	id(MEAN V)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
2	id(MEAN V)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
3	id(MEAN V)	[illegible]	[illegible]	[illegible]						
4	id(MEAN V)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

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Chemical: D Endpoint: PCT Tail DNA
Transformation: id Organ: Stomach

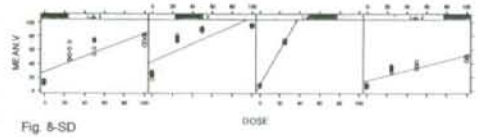

Fig. 8-SD

Table 9-SD

Chemical: D
Organ: Stomach Endpoint: PCT tail DNA
Transformation: id Link: id

Lab.	Variable	Slope	SE	L95%	U95%	P-value
1	id(MEAN.V)	0.57	0.09	0.381	0.759	0.00001
2	id(MEAN.V)	0.637	0.103	0.42	0.854	0.00001
3	id(MEAN.V)	2.528	0.036	2.444	2.612	0
4	id(MEAN.V)	0.384	0.046	0.269	0.46	0

Table 1-SE

Chemail: E Endpoint: PCT Tail DNA Transformation: id Organ: Stomach

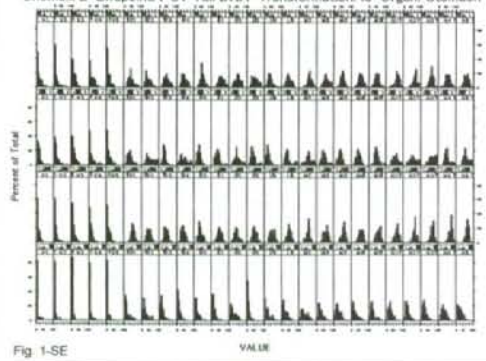

Fig. 1-SE

Chemail: E Endpoint: PCT Tail DNA
Transformation: id Organ: Stomach

Fig. 2-SE

Table 2-SE

Table 3-SE

EMS for chemical E Endpoint: %Tail DNA Effect: Difference
Transformation: ID Organ: Stomach

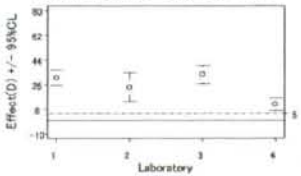

Fig. 3-SE

Table 4-SE

Chemical: E
Organ: Stomach Endpoint: PCT tail DNA
Transformation: id Link: id

Lab	Variable	Effect(D)	95%CL lower	95%CL upper
1	id(MEAN V)	31.02	25.27	36.78
2	id(MEAN V)	24.27	13.74	34.79
3	id(MEAN V)	33.44	27.02	39.87
4	id(MEAN V)	11.72	7.2	16.24

EMS for chemical E Endpoint: %Tail DNA Effect: Ratio
Transformation: ID Organ: Stomach

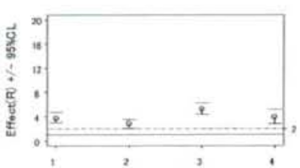

Fig. 4-SE

Table 5-SE

Chemical: E
Organ: Stomach Endpoint: PCT tail DNA
Transformation: id Link: id

Lab	Variable	Effect(R)	95%CL lower	95%CL upper
1	id(MEAN V)	3.71	2.98	4.8
2	id(MEAN V)	2.79	2.11	3.52
3	id(MEAN V)	5.29	4.45	6.25
4	id(MEAN V)	3.92	2.86	5.19

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Chemail: E Endpoint: PCT Tail DNA
Transformation: id Organ: Stomach

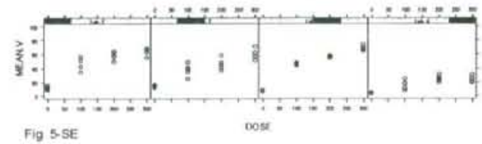


Fig. 5-SE

Table 6-SE

[illegible]

Lab	Variable	Dose	N	Mean	SD	Min	Max	Dose	N	Mean	SD	Min	Max	Dose	N	Mean	SD	Min	Max	Dose	N	Mean	SD	Min	Max
1	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
2	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
3	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
4	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Chemical: E Endpoint: %Tail DNA Effect: Difference
Transformation: ID Organ: Stomach

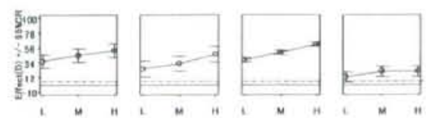


Fig. 6-SE

Table 7-SE

[illegible]

		V - L			V - M			V - H		
Lab	Variable	Effect(D)	95%CL lower	95%CL upper	Effect(D)	95%CL lower	95%CL upper	Effect(D)	95%CL lower	95%CL upper
1	id(MEAN V)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
2	id(MEAN V)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
3	id(MEAN V)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
4	id(MEAN V)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

24

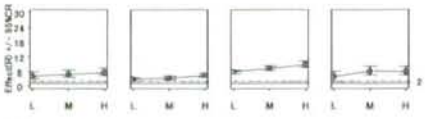


Fig. 7-SE

Table 8-SE

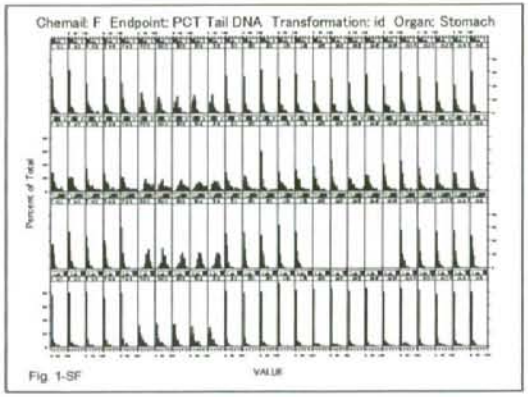


Fig. 1-SF

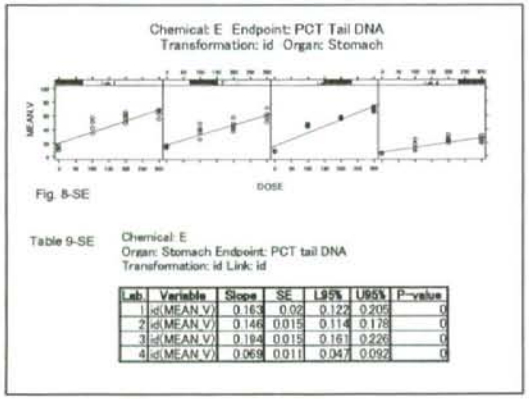


Fig. 8-SE

Table 9-SE

Chemical: E
Organ: Stomach Endpoint: PCT tail DNA
Transformation: id Link: id

Lab	Variable	Slope	SE	L95%	U95%	P-value
1	id(MEAN.V)	0.163	0.02	0.122	0.205	0
2	id(MEAN.V)	0.146	0.015	0.114	0.178	0
3	id(MEAN.V)	0.194	0.015	0.161	0.226	0
4	id(MEAN.V)	0.069	0.011	0.047	0.092	0

Table 1-SF

Chemail: F Endpoint: PCT Tail DNA
Transformation: id Organ: Stomach

Fig. 2-SF

Table 2-SF

Chemical: F
Organ: Stomach Endpoint: PCT tail DNA
Transformation: id Link: id

Lab.	Variable	Dose	N	Mean	SD	Min	Max	95%CL Low	95%CL Up
1	id(MEAN V)	[illegible]	5	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
2	id(MEAN V)	[illegible]	5	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
3	id(MEAN V)	[illegible]	5	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
4	id(MEAN V)	[illegible]	5	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Table 3-SF

Chemical: F
Organ: Stomach Endpoint: PCT tail DNA
Transformation: id Link: id

Lab.	Variable	Dose	N	Mean	SD	Min	Max
1	id(MEAN V)	[illegible]	5	[illegible]	[illegible]	[illegible]	[illegible]
2	id(MEAN V)	[illegible]	5	[illegible]	[illegible]	[illegible]	[illegible]
3	id(MEAN V)	[illegible]	5	[illegible]	[illegible]	[illegible]	[illegible]
4	id(MEAN V)	[illegible]	5	[illegible]	[illegible]	[illegible]	[illegible]

EMS for chemical F Endpoint: %Tail DNA Effect: Difference
Transformation: ID Organ: Stomach

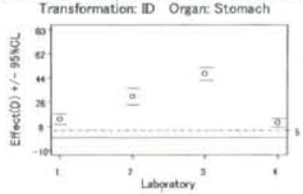


Fig. 3-SF

Table 4-SF

Chemical: F
Organ: Stomach Endpoint: PCT tail DNA
Transformation: id Link: id

Lab.	Variable	Effect(D)	95%CL lower	95%CL upper
1	id(MEAN V)	13.39	9.71	17.07
2	id(MEAN V)	30.21	24.13	36.29
3	id(MEAN V)	47.31	42.57	52.04
4	id(MEAN V)	10.86	7.56	14.16

EMS for chemical F Endpoint: %Tail DNA Effect: Ratio
Transformation: ID Organ: Stomach

Fig. 4-SF

Table 5-SF

Chemical: F
Organ: Stomach Endpoint: PCT tail DNA
Transformation: id Link: id

Lab.	Variable	Effect(R)	95%CL lower	95%CL upper
1	id(MEAN V)	2.27	1.79	3.06
2	id(MEAN V)	2.26	1.99	2.57
3	id(MEAN V)	6.77	5.94	7.78
4	id(MEAN V)	3.01	2.44	3.66

Chemail: F Endpoint: PCT Tail DNA
Transformation: id Organ: Stomach

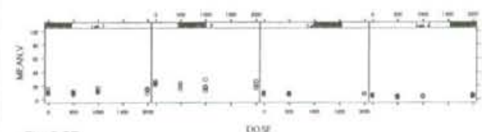


Fig. 5-SF

Table 6-SF

Chemical: F
Organ: Stomach Endpoint: PCT tail DNA
Transformation: id Link: id

Lab.	Variable	Vehicle						Low						Medium						High					
		Dose	N	Mean	SD	Min	Max	Dose	N	Mean	SD	Min	Max	Dose	N	Mean	SD	Min	Max	Dose	N	Mean	SD	Min	Max
1	id(MEAN V)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
2	id(MEAN V)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
3	id(MEAN V)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
4	id(MEAN V)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

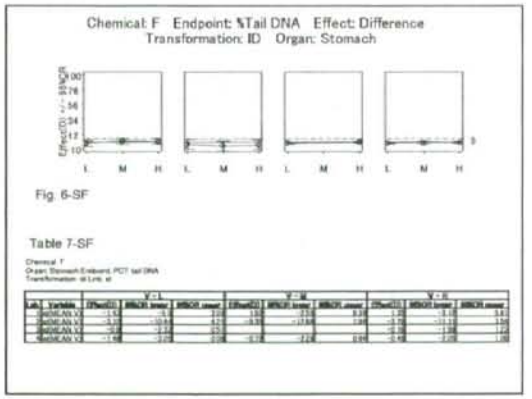


Fig. 6-SF

Table 7-SF

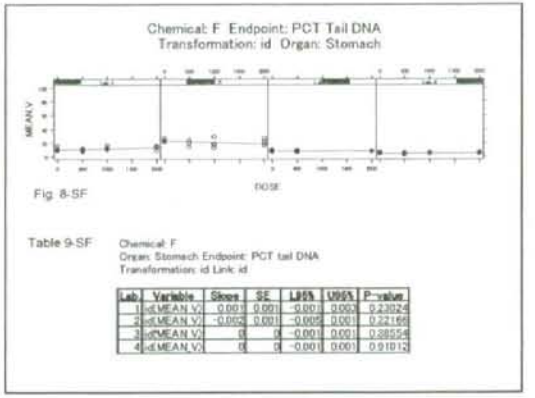


Fig. 8-SF

Table 9-SF

Chemical: F
Organ: Stomach Endpoint: PCT tail DNA
Transformation: id Link: id

Lab	Variable	Slope	SE	LB95	UB95	P-value
1	id:MEAN.V2	0.001	0.001	-0.001	0.003	0.23024
2	id:MEAN.V2	-0.002	0.001	-0.005	0.001	0.22166
3	id:MEAN.V2	0	0	-0.001	0.001	0.38554
4	id:MEAN.V2	0	0	-0.001	0.001	0.91012

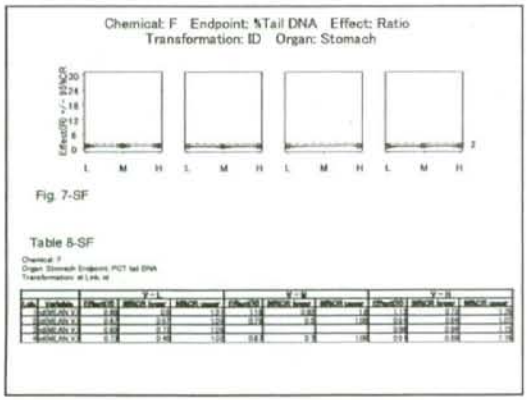


Fig. 7-SF

Table 8-SF

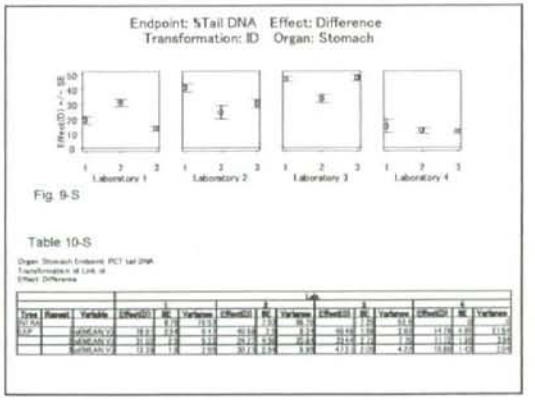


Fig. 9-S

Table 10-S

Endpoint: %Tail DNA Effect: Ratio
Transformation: ID Organ: Stomach

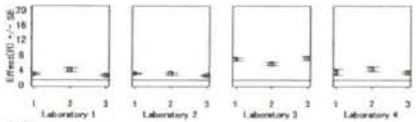


Fig. 10-S

Table 11-S

Organ: Stomach Endpoint: PCT tail DNA
Transformation: id Link: id
Effect: Ratio

			Lab.											
			1			2			3			4		
Type	Repeat	Variable	Effect(ID)	SE	Variance	Effect(ID)	SE	Variance	Effect(ID)	SE	Variance	Effect(ID)	SE	Variance
[illegible]				[illegible]	[illegible]		[illegible]	[illegible]		[illegible]	[illegible]		[illegible]	[illegible]
EXP	1	id(MEAN V)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	2	id(MEAN V)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
	3	id(MEAN V)	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

Table 12-S

Organ: Stomach Endpoint: PCT tail DNA
Transformation: id Link: id
Effect: Difference

Variable	Component		SE	Variance
id(MEAN V)	Lab.	–	12.58	158.33
	Repeat(Lab.)	–	6.86	47.05
	Residual	1-1	2.54	6.47
		1-2	2.5	6.23
		1-3	1.6	2.55
		2-1	2.5	6.24
		2-2	4.56	20.84
		2-3	2.64	6.95
		3-1	1.68	2.83
		3-2	2.79	7.76
		3-3	2.05	4.22
		4-1	4.65	21.64
		4-2	1.96	3.84
		4-3	1.43	2.04

Table 13-S

Organ: Stomach Endpoint: PCT tail DNA
Transformation: id Link: id
Effect: Ratio

Variable	Component		SE	Variance
id(MEAN V)	Lab.	–	1.66	2.75
	Repeat(Lab.)	–	0.43	0.19
	Residual	1-1	0.27	0.08
		1-2	0.45	0.2
		1-3	0.3	0.09
		2-1	0.23	0.05
		2-2	0.36	0.13
		2-3	0.15	0.02
		3-1	0.39	0.15
		3-2	0.46	0.21
		3-3	0.46	0.22
		4-1	0.72	0.52
		4-2	0.58	0.34
		4-3	0.31	0.1

Endpoint: PCT Tail DNA Transformation: id

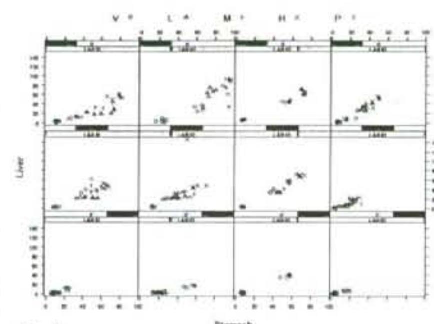


Fig. 11

31

32

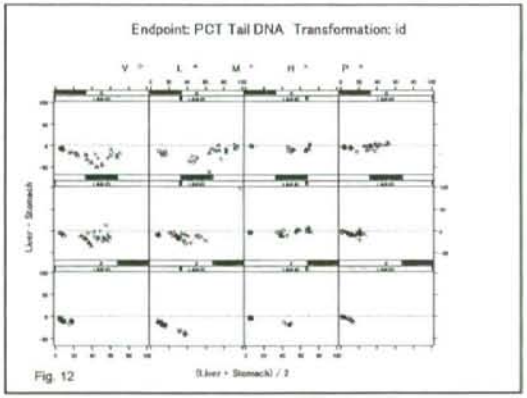


Fig. 12

Table 14

Endpoint: PCT tail DNA
Transformation: id Link: id

		Lab.			
		1	2	3	4
Chemical	Variable				
D	id(MEAN_V)	0.87	0.89	0.98	0.96
E	id(MEAN_V)	0.85	0.58	0.97	0.82
F	id(MEAN_V)	0.84	0.94	1	0.91

Comet: Osaka mtg.
Feb. 5, 2009

In Vivo Comet Assay:
Examination to Select Labs for 4th phase validation study

Examination to select labs for the next (4th phase) validation study

- Method: Check whether or not data of negative control and EMS groups meet the draft data acceptance criteria
- Test compound: EMS* and Two coded chemicals**
  - * Each exp. for coded chemicals included EMS group as a positive control, and thus 2 data of EMS/lab were expected to be available for judgment
  - ** Acrylamide & 2,6-diaminotoluene
- Protocol: version 12
- Result:
  - ✓Nine of 11 labs submitted the data to VMT
  - ✓Seven of nine labs passed the examination and were approved to participate in the next validation study
  - ✓Two of nine labs were requested to submit additional data to VMT, and, after that, they were approved

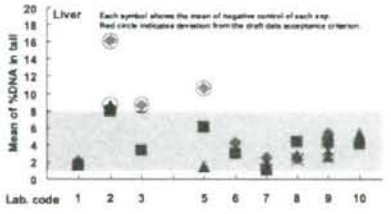
Data Acceptance Criteria (draft)*
based on 2nd phase validation study results

a. Negative control
- Mean of %DNA in tail in liver: 1-8%
- Mean of %DNA in tail in stomach: 1-30% (preferably 1-20%)

b. Positive control: EMS, 200 mg/kg, single (or twice) p.o.
- Effect (ratio of means of %DNA in tail between EMS & vehicle) in liver and stomach: 2-fold or higher
- Effect (difference of means of %DNA in tail between EMS & vehicle) in liver and stomach: 5% or higher
- CV of Effect (ratio) in two or more independent experiments with liver and stomach: 50% or less

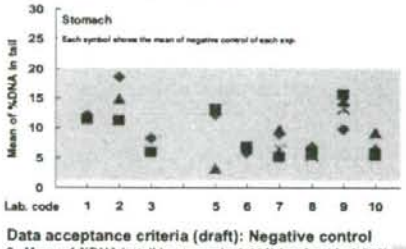
* Data acceptance criteria may be revised based on the 3rd phase validation results

Negative control ranges (%DNA in tail) in labs participating in the next validation



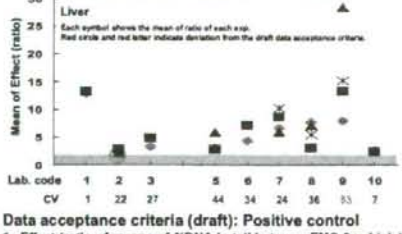
Data acceptance criteria (draft): Negative control
1. Mean of %DNA in tail in liver: 1-8%

Negative control ranges (%DNA in tail) in labs participating in the next validation



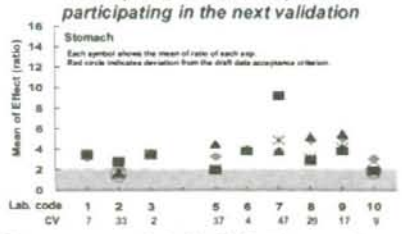
Data acceptance criteria (draft): Negative control
2. Mean of %DNA in tail in stomach: 1-30% (preferably 1-20%)

Effect (ratio, %DNA in tail) in labs participating in the next validation



Data acceptance criteria (draft): Positive control
1. Effect (ratio of means of %DNA in tail between EMS & vehicle) in liver and stomach: 2-fold or higher
2. CV of Effect (ratio) in two or more independent experiments with liver and stomach: 50% or less

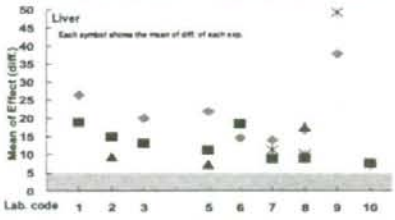
Effect (ratio, %DNA in tail) in labs participating in the next validation



Data acceptance criteria (draft): Positive control

1. Effect (ratio of means of %DNA in tail between EMS & vehicle) in liver and stomach: 2-fold or higher
2. CV of Effect (ratio) in two or more independent experiments with liver and stomach: 50% or less

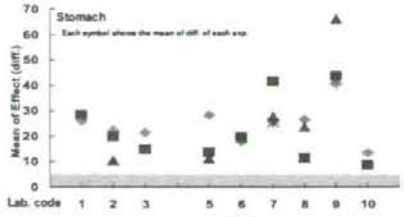
Effect (difference, %DNA in tail) in labs participating in the next phase validation



Data acceptance criteria (draft): Positive control

3. Effect (difference of means of %DNA in tail between EMS & vehicle) in liver and stomach: 5% or higher

Effect (difference, %DNA in tail) in labs participating in the next phase validation



Data acceptance criteria (draft): Positive control

3. Effect (difference of means of %DNA in tail between EMS & vehicle) in liver and stomach: 5% or higher

Facilities and Representatives of 4th Phase Validation Study

- Alphabetic order (not related to lab code) -

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

° Leading laboratory

Comet: Osaka mtg.
Feb. 5, 2009

In Vivo Comet Assay: 4th Phase Validation Study

Facilities and Representatives of 4th Phase Validation Study

- Alphabetic order -

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

* Leading laboratory

Purpose of 4th Phase Validation Study

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

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

Draft: Outline and Schedule of 4th Phase Validation Study

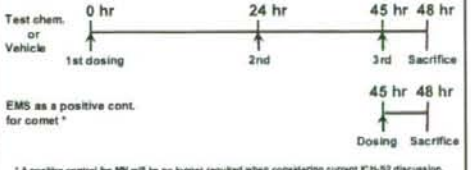
- ✓ The study will be performed in accordance with the version 14 study protocol and the study plan*.
  * This will be prepared for each validation study to define the purpose and specific points.
- ✓ Max. 4 coded test chemicals will be examined in each lab.
- ✓ Labs will have one year as a net experimental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

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

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

Modification of Administration Regimen

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



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

Points to be discussed related to the modification of administration regimen

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

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

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

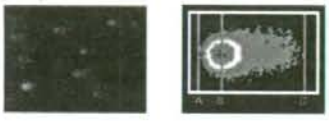
- ✓ We are now planning to have a workshop of this validation study as one session of the International Comet Assay Workshop in August. At the workshop, the most important topic will be the image analysis, and we will explain how to distinguish between Comet and Hedgehog based on the discussion in this Osaka mtg.. We will invite all participants of this validation study to the workshop, especially who could not participate in this Osaka mtg..
- ✓ Labs that the representative has attended this Osaka mtg. will hopefully finish the first experiment by July. We will check the data quickly before the above workshop. If we feel necessity of discussion on the data, we will plan to have a mtg. at the workshop.
- ✓ Labs that the representative could not attended this Osaka mtg. will also start the first experiment, but VMT may direct them to pause the experiment at the dehydration of slides. After they attend the above workshop and know how to analyze the image, they will start the image analysis.

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

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

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

International Validation Study of *in* Vitro Alkaline Comet Assay



Masamitsu Honma

Division of Genetics and Mutagenesis
National Institute of Health Sciences

Purpose of the Validation Study

In order to establish a robust in vitro Comet assay protocol and to make consensuses for evaluation and interpretation of the Comet results (including cytotoxicity), leading laboratories conduct the in vitro Comet assay for several genotoxic or non-genotoxic chemicals. The management members review and validate the Comet results with the consultation of experts. Form the studies, we pursuit the possibility of the in vitro Comet assay as alternative for other in vitro or in vivo genotoxicity tests.

Organization (2008.3~)

Validation Management Team (VMT)
M. Hayashi (Chair, JaCVAM/An-Pyo. Ctr.)
R. Corvi (ECVAM)
M. Honma (NIHS)
Y. Uno (MTPC, JEMS/MMS)
L. Schechtman (Consultant)
R. Tice (NIEHS)

Secretariat
H. Kojima (JaCVAM/NIHS)

Leading laboratory
K. Yamakage (FDSC, JP)
P. Escober (Boehringer-Ingelheim, USA)
K. Pant (Bio-Reliance, USA)
B. Burlinson (HLS, UK)
A. Kraynak (Merk, USA)

Consultation Team
N. Asano (Nitto Denko, JEMS/MMS)
D. Lovell (Univ. of Surrey)
T. Morita (NIHS)
N. Nakashima (PMDA)
Y. Ohno (JaCVAM/NIHS)
T. Omori (Kyoto Univ.)
YF. Sasaki (Hachinohe Nat.Coll.Tech.)
M. Suzuki (An-Pyo Ctr.)
S. Hoffman (ECVAM)
G. Speit (Univ. of Ulm)
A. Collins (Unv. of Oslo)
S. Park (KFDA)
Y. Seo (Kyung Hee Unv.)

Local Committee in JPN
Mainly from JEMS/MMS members

Specific Issues of the In Vitro Comet Assay Protocol

1. Cells, Cell lines
   - Suspension cells vs. adherent cells
2. Duration of treatment with chemicals
   - Short vs. long
3. Dose selection
   - Cytotoxic parameter, level of cytotoxicity
4. Metabolic activation
   - S9 condition
5. Relevance to other genotoxic responses
   - Comparison of sensitivity and reactivity with other genotoxicity tests
6. Statistical analysis

Basic Protocol for Alkaline Comet Assay

* In vitro alkaline Comet assay protocol is identical after cell preparation.

		In Vivo Comet Standard Procedure
Agarose gel and sample preparation	Bottom gel	1.0-1.5% low-gelling temperature-agarose in PBS (4 ml/slide)
	Sample gel (A)	0.5% low-gelling temperature-agarose in PBS
	Solution of suspended cells (B)	Cells in HBSS with 20 mM EDTA and 10% DMSO*
	Mixture Final conc. of agarose	(A):(B)= 3:1 0.45%
Lysis and electroporation	Lysis solution	2.5M NaCl, 100mM Na2EDTA, 10mM Tris-base, 10% DMSO, 1% Triton X (pH 10) *
	Lysis condition	Overnight, 4C
	Rinse solution/ Condition	Distilled water/ Dipping
	Electrophoresis solution	0.3M NaOH, 1mM EDTA (pH>13), <10C
	Electrophoresis condition	Unwinding 20min) + Electrophoresis 0.7-1 V/cm (300mA), < 10C
Staining	Neutralization/ Dehydration	0.4M Tris-base (pH 7.5) at least 5 min/ Absolute ethanol at least 2 min
	Staining dye/ Time	SYBR Gold/ 30 min
Scoring and analysis	Comet analysis	Comet IV, Tail length, Tail moment, % tail DNA

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

Phase I Study (2007.10~)

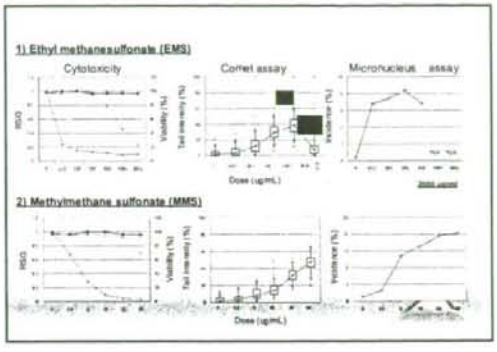
Experimental Condition

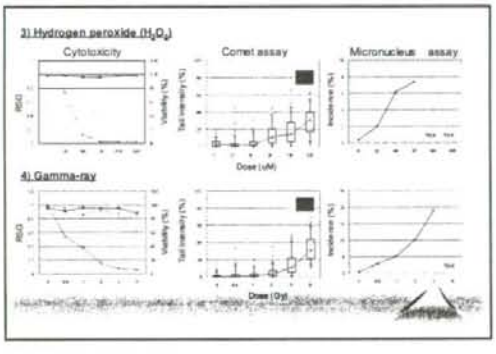
1. Cell lines
   - TK6 (Same lot of cells are supplied from ATCC)
2. S9
   - Rat induced liver S9
3. Treatment time
   - 4h
4. Cytotoxic parameter
   1) Trypan blue dye exclusion test just after the treatment (RS)
   2) Relative cell growth for 24 h after the treatment (RSG)
4. Comet analysis
   - According to the in vivo protocol
5. Measuring Comet
   - SYBR gold staining
   - Comet IV, Tail length, Tail moment, Tail intensity, 100 cells/dose/tube
6. Statistical analysis
   - Dr. Suzuki is developing

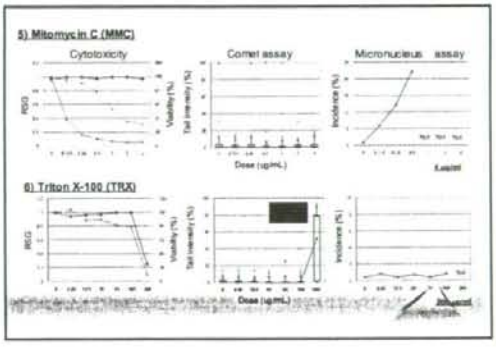
Test Chemicals

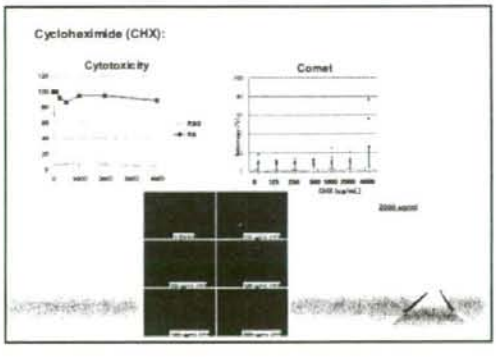
1. Ethymethansulfonate (EMS): alkylating agent (DMSO)
   S9-mix +/-: 0, 62.5, 125, 250, 500, 1000, 2000 ug/ml
2. Mitomycine C (MMC): cross-linker (physiological saline)
   S9-mix +/-: 0, 0.125, 0.25, 0.5, 1, 2, 4 ug/ml
3. 2-Aminoanthracene (2AA): aromatic hydrocarbon (DMSO)
   S9-mix -: 0, 125, 250, 500, 1000, 2000, 4000 ug/ml
   S9-mix +: 0, 0.125, 0.25, 0.5, 1, 2, 4 ug/ml
4. Cycloheximide (CHX): inhibitor for protein synthesis (ethanol)
   S9-mix +/-: 0, 62.5, 125, 250, 500, 1000, 2000 ug/ml
5. Triton-X (TRX): detergent (physiological saline)
   S9-mix +/-: 0, 6.25, 12.5, 25, 50, 100, 200 ug/ml

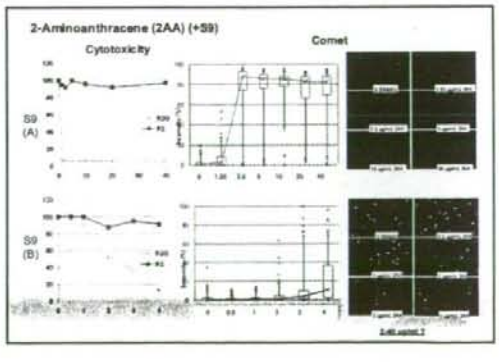
*Because test chemicals are supplied from VNT, every laboratory examine same lot of chemicals.











2008. Oct.1 Phase I, Validation meeting
In Osaka

Data analysis and statistical analysis of phase I study
by Masaya Suzuki
(An-Pyo Center, Japan)

Phase II Study (2008.8~)

Experimental Condition (I)

Top concentration and dose selection

- The laboratory should conduct the Comet assay for the chemicals until 5mg/ml if no cytotoxicity is observed. When cytotoxicity is observed less than 5mg/ml, a top concentration is determined by cytotoxicity tests. The top concentration should show enough cytotoxicity, but not severe cell damage causing a lot of non-detectable cell nuclei (NDCN; hedgehog). The recommended top concentration is one with 80% TBDE, 20% NDCN, or no cell growth for 24 h after the treatment. The laboratory can conduct preliminary experiment for the dose-finding.
- Each main experiment usually consists of one solvent control and at least five concentration of the test chemical. As a rule, 2-fold serial dilutions were prepared from the top concentration.

Phase II Study (2008.8~)

Experimental Condition (II)

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.

Phase II Study (2008.8~)

Test Chemicals and solvent

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

Phase II Study (2008.8~)

Progress (6 chemicals)

Boehringer-Ingelheim, USA	- Completed
Bio-Reliance, USA	- Completed
HLS, UK	- Completed
FDSC, JP	- Four chemicals left
Merk, USA	- Withdraw

Action Plan for In Vitro Validation Study

2009.3	Collecting data of the 2nd phase in vitro Comet assay.
2009.5	Data analysis and interim meeting of the 2nd phase in vitro Comet assay.
2009.8	VMT meeting for validating the 2nd phase in vitro Comet assay.
2009.11	The 3rd phase study will start.

Data analysis and statistical analysis of phase I study

Masaya Suzuki
Feb. 4th 2009

History of In-vitro comet assay (JACVAM validation study)

- 2008. Jan. In-vitro comet assay phase I validation start
- 2008. Oct. Internal VMT meeting
  The result of phase I validation study was shown.
- 2008. Nov.
  deadline for receive data (phase II validation)
  we didn't receive the datasheets completely due to be delay to send the test-substance from VMT.
- 2009. Feb. (Now)
  We are collecting the datasheets
  therefore we have not finished statistical analyses completely.

Overview of in-vitro phase I validation study

- 5 participating facilities : BSRC, Bio-Reliance, HLS, Merk, FDSC
- Test substances were 5, EMS, MMC, 2AA,CHD,TRX (unmasked)

[Description of protocol about test substances]
For EMS, MMC, 2AA, these concentrations cover concentrations showing enough cytotoxicity and positive responses in the TK-gene mutation assay in TK6 cells (Honma et al., 1994 and unpublished data). The top concentrations are more than 10-folds of those in the TK-mutation assay. CHD did not show any cytotoxicity and Comet-responses in TK6 cells even at the highest concentrations (5000 ug/ml) (Heddestrom et al., 1998). Although the in vitro cytotoxicity of TRX in TK6 cells are not clear, it exhibits clear cytotoxicity less than at 200 uM (50 ug/ml) in A549 cells (Vock et al., 1998). We don't take positive control even for experiments with non-genotoxic chemicals. (The reference from protocol.)

- Cell line:TK6 Human lymphoblast cell line
- In each dose the cell line were divided into 2 tubes.
  2 slides were made from each tube
  50 cells were on a slide (total number of data is usually 200 in each dose.)
- Candidate variables for analysis Tail moment (TM), Tail length (TL), and % tail DNA (TD)
- The protocol of in-vitro study was almost described according to that of the in-vivo validation study which was previously performed.

Consideration of statistical analyses from phase I in-vitro comet assay (before analysis)

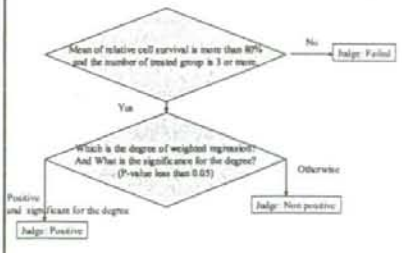
- Although pair-wise comparison (such as between control group and each dose group, Dunnett test) can be performed based on cell unit or tube unit, the pair-wise comparison can not be performed based on cell line unit. (the number of cell line is 1.)
- Comet IV system generates 100 items.
  3 items values such as tail moment ,tail length and % tail DNA are gathering for in-vitro comet validation.
  Finally we debated (% tail DNA) value is primary analysis variable according to in-vivo study decision.

Consideration for the judgment of the comet assay in-vitro Study in the meeting on Oct.1st

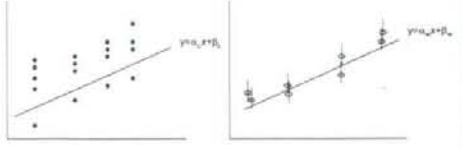
- In the dosed groups, when relative cell survival is more than 80%, The data in that group will be included for the statistical analyses.
  The statistical analysis could be performed, when the number of dose satisfied with above condition group is 3 or more.
- Weighted regression will be performed with mean value of each group (explanatory variable) and 1/(standard error of the mean) of each group (weighted value).

  The statistical test for the existence for the degree of regression analysis will be performed. When p-value of the statistical test is less than 5%, we decided that the degree is significantly existence.

Proposal for the candidate flow of judgment



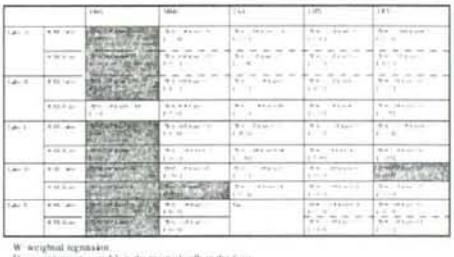
Regression analysis



Regression analysis (common)　　Regression analysis (weighted)

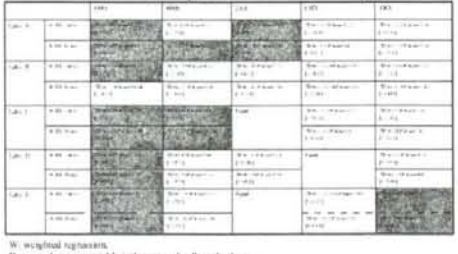
We could not apply the common regression analysis
since there are even if 1 data for analysis in per dose in in-vitro I[st] validation study
Therefore, We applied the weighted regression analysis
(We set mean value to explanatory value and 1/(standard error) to weighted value.)

The Result of weighted regression analysis (-S9,TD, phase I validation study)



W: weighted regression
Dose: explanatory variable is the mean of cells in the dose
Tube: explanatory variable is the mean of cells in the each tube
When the test substance is positive, the background of the result is indicated with

The Result of weighted regression analysis (+S9,TD , phase I validation study)



W: weighted regression
Dose: explanatory variable is the mean of cells in the dose
Tube: explanatory variable is the mean of cells in the each tube
When the test substance is positive, the background of the result is indicated with

Summary in Phase1 validation study

- Multiple comparison such as Dunnett test, based on cell unit are tend to increase significance because sample number (number of cell) is too large. (Data not shown)
- The number of data is 1 in each dose (only one cell line was used), statistical comparison is difficult to detect the significant difference.
- We performed the 2 type of weighted regression (tube unit and dose unit, explanatory value is mean and weight is 1/(standard error)) and compared to results. As the result, the weighted regression based on tube unit is more matched to the biological empirical estimation.
- We applied this methodology to the phase II in-vitro validation study, we hope that more sophisticated protocol will be composed in the future.

背景

- 変異原性の判定に有意差検定が用いられることが多い.
- In vivo comet assayでは,各動物単位の% Tail DNAの平均値を用いた統計手法を検討している.
- In vivo comet assayと同一単位の平均値で解析することが好ましいと考え,In vitro comet assayでは細胞株単位の統計手法を検討した.
- 群間比較を行う統計学検定は,各群2例以上を必要とするため,In vitro comet assayのデータについて適応できない.
- 今回,各群のデータ数が1の場合に判定できる重み付回帰分析をPhase I validationのデータに適応した.

背景(続き)

- Phase Iの解析結果から,一部のデータについて,実測値と回帰直線の間に乖離の可能性が見られた.

  今回 用量を変数変換しない場合と常用対数変換した場合の回帰直線を比較した.

材料と方法

- In vitro comet assayのphase I validationで得られた% Tail DNA(TD)のデータを使用した.
- 陰性対照群を除くDose(変数変換なし)と,Log (Dose) (常用対数変換)を用いて,重み付き回帰直線を作成した.

  説明変数として,各用量のTDの平均値または各TubeのTDの平均値を用い,それぞれの標準誤差の逆数を重みとして用いた.
- 予測値と実測値の差(残差)の平方和を比較し,残差平方和が低い回帰直線を実測値がよく当てはまった直線であると判定した.

結果

説明変数	変数変換	
	なし	常用対数
各用量のTDの平均値	25	21
各TubeのTDの平均値	26	20

結果

- 残差和が低い回帰直線の頻度を集計した.
- 各説明変数とも,変数変換しない回帰直線の数が多かった.