Antagonist ANOVA Results for Intralaboratory Comparison of Reference Standard & Controls

	p-value[1,2,3]	F value[4]
DMSO	<0.001	41.8
Fold-Reduction	<0.001	29.2
E2 Control	<0.001	134.7

[1]Variability is statistically significant at p<0.05.
[2]ANOVA analyzed values from the three participating laboratories.
[3]Values in italics have p values that are less than 0.05.
[4]A ratio of between-laboratory variability to within-laboratory variability - a ratio of 1.0 indicates that the within-laboratory variability is equal and a ratio of zero indicates that all means are equal.

Newman-Keuls - Interlaboratory Comparison of Antagonist Reference Standard & Controls

	DMSO[1,2,3]		Fold-Reduction[2,3,4]		E2 Control [2,3,5]	
	Mean Difference	P value	Mean Difference	p value	Mean Difference	P value
XDS vs ECVAM	2228	<0.001	4.7	<0.001	918	<0.01
XDS vs Hypothl	4190	<0.001	8.5	<0.05	2989	<0.001
ECVAM vs Hypothl	1961	<0.001	4.2	<0.001	3904	<0.001

[1]Presented in relative light units
[2]Variability is statistically significant at p<0.05
[3]Values in italics have p values that are less than 0.05
[4]Presented in fold-reduction
[5]Presented in adjusted and normalized relative light units

The LUMI-CELL® ER Assay Validation Study - Phase IIb

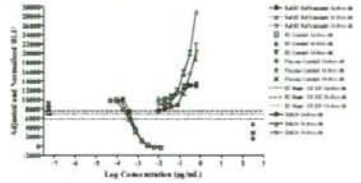
Intra- and Inter-laboratory Reproducibility of Testing Results for Antagonist Substances

Antagonist Comprehensive Testing - Concentrations Tested & Cell Viability for API

Substance Name	XDS		ECVAM		Hypothl	
	Concentration (log(M))	Cell Viability[1]	Concentration (log(M))	Cell Viability[1]	Concentration (log(M))	Cell Viability[1]
API	-5.00 x 10^-5	3	5.00 x 10^-5	1	5.00 x 10^-5	1
	2.50 x 10^-5	3	2.50 x 10^-5	1	2.50 x 10^-5	1
	1.25 x 10^-5	3	1.25 x 10^-5	1	1.25 x 10^-5	1
	6.25 x 10^-6	1	6.25 x 10^-6	1	6.25 x 10^-6	1
	3.13 x 10^-6	1	3.13 x 10^-6	1	3.13 x 10^-6	1
	1.56 x 10^-6	1	1.56 x 10^-6	1	1.56 x 10^-6	1
	7.81 x 10^-7	1	7.81 x 10^-7	1	7.81 x 10^-7	1
	3.91 x 10^-7	1	3.91 x 10^-7	1	3.91 x 10^-7	1
	1.95 x 10^-7	1	1.95 x 10^-7	1	1.95 x 10^-7	1
	9.77 x 10^-8	1	9.77 x 10^-8	1	9.77 x 10^-8	1

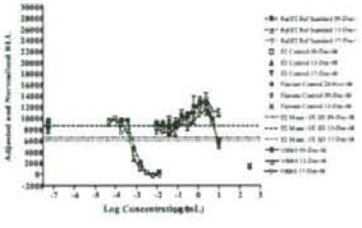
[1]The cell viability score indicates the score that was given for that concentration on all plates tested.

Phase IIb Antagonist Results for API at XDS

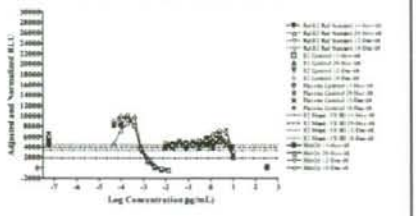


Test concentrations that were cytotoxic (cell viability scores of 2 or greater) were excluded from the graph because they are not used in the determination of antagonist activity.

Phase IIb Antagonist Results for API at ECVAM



Phase IIb Antagonist Results for API at Hiyoshi



Interlaboratory Comparison of API Antagonist Activity

	Plates Tested	Plates Testing Positive for Antagonism[1]	Plates Testing Negative for Antagonism[1]	ICCVAM Meta-data[2]
XDS	3	0	3	#
ECVAM	3	2	1	
Hiyoshi	4	3	1	

[1] If mean adjusted RLU values for a given concentration(s) of test substance is less than the mean minus three times the standard deviation of the plate E2 control values, the test substance is considered positive for antagonist activity – any response above this threshold is considered negative for antagonist activity.

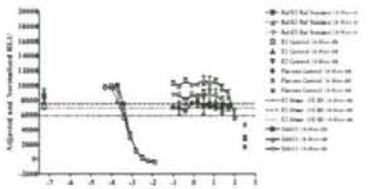
[2] # indicates that the substance was positive in the single assay in which it was tested.

Antagonist Comprehensive Testing - Concentrations Tested & Cell Viability for ATZ

Substance Name	XDS Concentration (pg/mL)	XDS Cell Viability[1]	ECVAM Concentration (pg/mL)	ECVAM Cell Viability[1]	Hiyoshi Concentration (pg/mL)	Hiyoshi Cell Viability[1]
ATZ	1.00 x 10^5	2 / 2	1.00 x 10^5	1	1.00 x 10^5	1
	5.00 x 10^4	2 / 1	5.00 x 10^4	1	5.00 x 10^4	1
	2.50 x 10^4	1	2.50 x 10^4	1	2.50 x 10^4	1
	1.25 x 10^4	1	1.25 x 10^4	1	1.25 x 10^4	1
	6.25 x 10^3	1	6.25 x 10^3	1	6.25 x 10^3	1
	3.13 x 10^3	1	3.13 x 10^3	1	3.13 x 10^3	1
	1.56 x 10^3	1	1.56 x 10^3	1	1.56 x 10^3	1
	7.81 x 10^2	1	7.81 x 10^2	1	7.81 x 10^2	1
	3.91 x 10^2	1	3.91 x 10^2	1	3.91 x 10^2	1
	1.95 x 10^2	1	1.95 x 10^2	1	1.95 x 10^2	1
	9.77 x 10^1	1	9.77 x 10^1	1	9.77 x 10^1	1

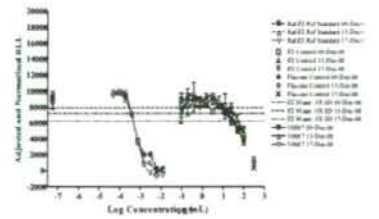
[1] Multiple scores indicate the cell viability scores for concentrations tested in each plate when scores differed from plate to plate. A single cell viability score indicates the score that was given for that concentration on all plates tested.

Phase IIb Antagonist Results for ATZ at XDS

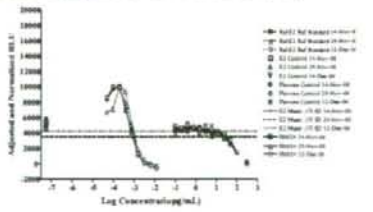


Test concentrations that were cytotoxic (cell viability scores of 2 or greater) were excluded from the graph because they are not used in the determination of antagonist activity.

Phase IIb Antagonist Results for ATZ at ECVAM



Phase IIb Antagonist Results for ATZ at Hiyoshi



Interlaboratory Comparison of ATZ Antagonist Activity

	Plates Tested	Plates Testing Positive for Antagonism[†]	Plates Testing Negative for Antagonism[†]	ICCVAM Meta-data[‡]
XDS	3	1	2	
ECVAM	3	3	0	
Hiyoshi	3	3	0	

[†] Mean adjusted RLU values for a given concentration(s) of test substance is less than the mean minus three times the standard deviation of the plate E2 control values; the test substance is considered positive for antagonist activity - any response above this threshold is considered negative for antagonist activity.

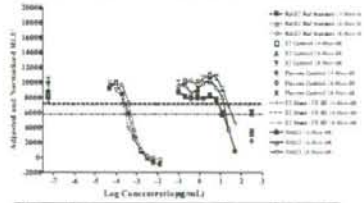
[‡] - indicates that the substance was uniformly negative in multiple assays

Antagonist Comprehensive Testing - Concentrations Tested & Cell Viability for BBP

Substance Name	XDS Concentration (log10 M)	XDS Cell Viability[†]	ECVAM Concentration (log10 M)	ECVAM Cell Viability[†]	Hiyoshi Concentration (log10 M)	Hiyoshi Cell Viability[†]
BBP	-4.00 x 10^-1	2.3.3.	-4.00 x 10^-1	1	-4.00 x 10^-1	1
	-5.00 x 10^-1	1.2.1	-5.00 x 10^-1	1	-5.00 x 10^-1	1
	-6.00 x 10^-1	1	-6.00 x 10^-1	1	-6.00 x 10^-1	1
	-7.00 x 10^-1	1	-7.00 x 10^-1	1	-7.00 x 10^-1	1
	-8.00 x 10^-1	1	-8.00 x 10^-1	1	-8.00 x 10^-1	1
	-9.13 x 10^-1	1	-9.13 x 10^-1	1	-9.13 x 10^-1	1
	-1.04 x 10^0	1	-1.04 x 10^0	1	-1.04 x 10^0	1
	-1.41 x 10^0	1	-1.41 x 10^0	1	-1.41 x 10^0	1
	-2.87 x 10^0	1	-2.87 x 10^0	1	-2.87 x 10^0	1
	-1.66 x 10^1	1	-1.66 x 10^1	1	-1.66 x 10^1	1
	-8.77 x 10^1	1	-8.77 x 10^1	1	-8.77 x 10^1	1

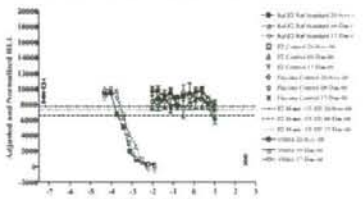
[†] Multiple scores indicate the cell viability scores for concentrations tested in each plate when scores differed from plate to plate. A single cell viability score indicates the score that was given for that concentration on all plates tested.

Phase IIb Antagonist Results for BBP at XDS

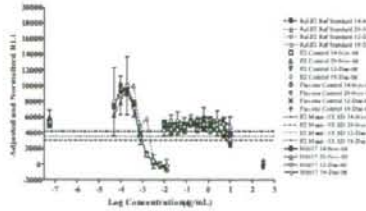


Test concentrations that were cytotoxic (cell viability scores of 2 or greater) were excluded from the graph because they are not used in the determination of antagonist activity

Phase IIb Antagonist Results for BBP at ECVAM



Phase IIb Antagonist Results for BBP at Hiyoshi



Interlaboratory Comparison of BBP Antagonist Activity

	Plates Tested	Plates Testing Positive for Antagonism[†]	Plates Testing Negative for Antagonism[†]	ICCVAM Meta-data[‡]
XDS	3	3	0	
ECVAM	3	2	1	
Hiyoshi	4	2	2	

[†] Mean adjusted RLU values for a given concentration(s) of test substance is less than the mean minus three times the standard deviation of the plate E2 control values; the test substance is considered positive for antagonist activity - any responses above this threshold is considered negative for antagonist activity.

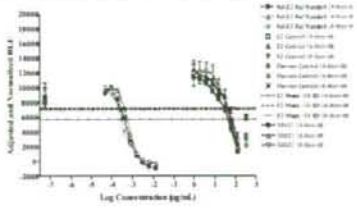
[‡] - indicates that the substance was uniformly negative in multiple assays

Antagonist Comprehensive Testing - Concentrations Tested & Cell Viability for CORT

Substance Name	XDS Concentration (g/mL)	XDS Cell Viability[1]	ECVAM Concentration (g/mL)	ECVAM Cell Viability[1]	Hiyoshi Concentration (g/mL)	Hiyoshi Cell Viability[1]
CORT	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

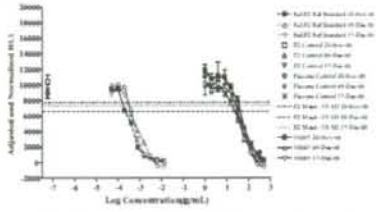
[1]Multiple scores indicate the cell viability scores for concentrations tested in each plate when scores differed from plate to plate. A single cell viability score indicates the score that was given for that concentration on all plates tested.

Phase IIb Antagonist Results for CORT at XDS



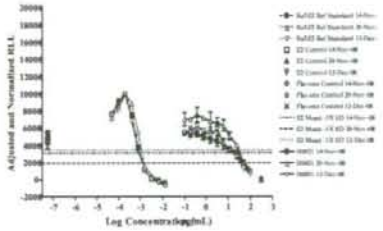
Test concentrations that were cytotoxic (cell viability scores of 2 or greater) were excluded from the graph because they are not used in the determination of antagonist activity.

Phase IIb Antagonist Results for CORT at ECVAM



Test concentrations that were cytotoxic (cell viability scores of 2 or greater) were excluded from the graph because they are not used in the determination of antagonist activity.

Phase IIb Antagonist Results for CORT at Hiyoshi



Interlaboratory Comparison of CORT Antagonist Activity

	Plates Tested	Plates Testing Positive for Antagonism[1]	Plates Testing Negative for Antagonism[1]	ICCVAM Meta-data[2]
XDS	3	3	0	-
ECVAM	3	3	0	
Hiyoshi	3	3	0	

[1] If mean adjusted RLU values for a given concentration(s) of test substance is less than the mean minus three times the standard deviation of the plate EJ control values, the test substance is considered positive for antagonist activity - any response above this threshold is considered negative for antagonist activity.

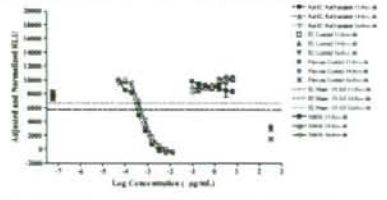
[2] - indicates that the substance was uniformly negative in multiple assays

Antagonist Comprehensive Testing - Concentrations Tested & Cell Viability for DDT

Substance Name	XDS Concentration (g/mL)	XDS Cell Viability[1]	ECVAM Concentration (g/mL)	ECVAM Cell Viability[1]	Hiyoshi Concentration (g/mL)	Hiyoshi Cell Viability[1]
DDT	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]

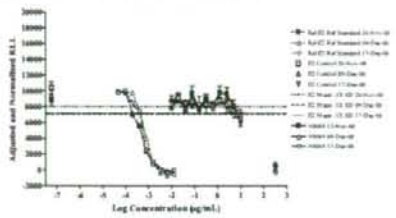
[1]The cell viability score indicates the score that was given for that concentration on all plates tested.

Phase IIb Antagonist Results for DDT at XDS

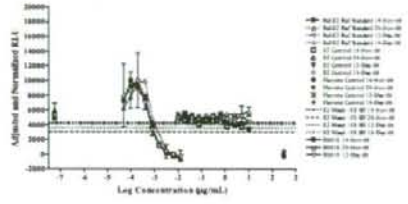


Test concentrations that were cytotox-IC (cell viability scores of 2 or greater) were excluded from the graph because they are not used in the determination of antagonist activity.

Phase IIb Antagonist Results for DDT at ECVAM



Phase IIb Antagonist Results for DDT at Hiyoshi



Interlaboratory Comparison of DDT Antagonist Activity

	Plates Tested	Plates Testing Positive for Antagonism[1]	Plates Testing Negative for Antagonism[1]	ECVAM Meta-data[2]
XDS	3	0	3	+
ECVAM	3	2	1	
Hiyoshi	4	3	2	

[1] Mean adjusted RLU values for a given concentration(s) of test substance is less than the mean minus three times the standard deviation of the plate EC control values; the test substance is considered positive for antagonist activity - any responses above this threshold is considered negative for antagonist activity.

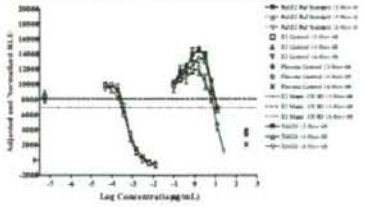
[2] Indicates that the substance was positive in the single assay in which it was tested.

Antagonist Comprehensive Testing - Concentrations Tested & Cell Viability for FLA

Reference Name	XDS		ECVAM		Hiyoshi	
	Concentration (µg/mL)	Cell Viability[1]	Concentration (µg/mL)	Cell Viability[1]	Concentration (µg/mL)	Cell Viability[1]
FLA	1.56 x 10^{-1}	4	1.56 x 10^{-1}	4P	1.56 x 10^{-1}	1
	6.25 x 10^{-2}	2,2,2	6.25 x 10^{-2}	1	6.25 x 10^{-2}	1
	2.50 x 10^{-2}	2,2,1	2.50 x 10^{-2}	1	2.50 x 10^{-2}	1
	1.00 x 10^{-2}	1	1.00 x 10^{-2}	1	1.00 x 10^{-2}	1
	4.00 x 10^{-3}	1	4.00 x 10^{-3}	1	4.00 x 10^{-3}	1
	1.60 x 10^{-3}	1	1.60 x 10^{-3}	1	1.60 x 10^{-3}	1
	6.40 x 10^{-4}	1	6.40 x 10^{-4}	1	6.40 x 10^{-4}	1
	2.56 x 10^{-4}	1	2.56 x 10^{-4}	1	2.56 x 10^{-4}	1
	1.02 x 10^{-4}	1	1.02 x 10^{-4}	1	1.02 x 10^{-4}	1
	4.10 x 10^{-5}	1	4.10 x 10^{-5}	1	4.10 x 10^{-5}	1

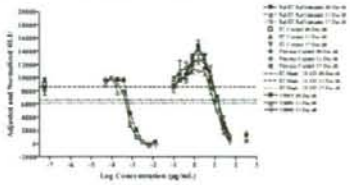
[1] Multiple scores indicate the cell viability scores for concentrations tested in each plate when scores differed from plate to plate. A single cell viability score indicates the score that was given for that concentration on all plates tested.

Phase IIb Antagonist Results for FLA at XDS



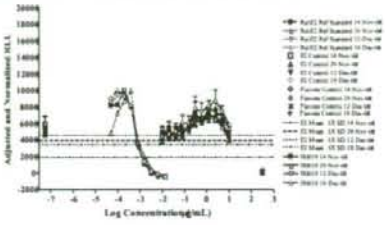
Test concentrations that were cytotox-IC (cell viability scores of 2 or greater) were excluded from the graph because they are not used in the determination of antagonist activity.

Phase IIb Antagonist Results for FLA at ECVAM



Test concentrations that were cytotoxic (cell viability scores of 2 or greater) were excluded from the graph because they are not used in the determination of antagonist activity.

Phase IIb Antagonist Results for FLA at Hiyoshi



Interlaboratory Comparison of FLA Antagonist Activity

	Plates Tested	Plates Testing Positive for Antagonism[1]	Plates Testing Negative for Antagonism[1]	ICCVAM Meta-data[2]
XDS	3	3	0	
ECVAM	3	3	0	
Hiyoshi	4	0	4	

[1] If mean adjusted RLU values for a given concentration(s) of test substance is less than the mean minus three times the standard deviation of the plate C2 control values, the test substance is considered positive for antagonist activity - any response above this threshold is considered negative for antagonist activity.

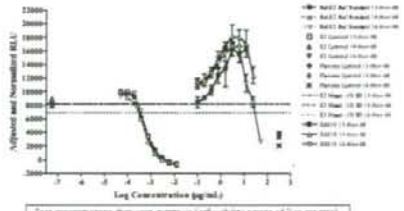
[2] IIIIII indicates that the substance was uniformly positive in multiple assays.

Antagonist Comprehensive Testing - Concentrations Tested & Cell Viability for GEN

Substance Name	XDS Concentration (kg/mL)	XDS Cell Viability[1]	ECVAM Concentration (kg/mL)	ECVAM Cell Viability[1]	Hiyoshi Concentration (kg/mL)	Hiyoshi Cell Viability[1]
GEN	1.00 x 10^{-3}	0.0 0	1.00 x 10^{-3}	1	1.00 x 10^{-3}	1
	5.00 x 10^{-4}	0.2 1	5.00 x 10^{-4}	1	5.00 x 10^{-4}	1
	2.50 x 10^{-4}	0 1 1	2.50 x 10^{-4}	1	2.50 x 10^{-4}	1
	1.25 x 10^{-4}	1	1.25 x 10^{-4}	1	1.25 x 10^{-4}	1
	6.25 x 10^{-5}	1	6.25 x 10^{-5}	1	6.25 x 10^{-5}	1
	3.13 x 10^{-5}	1	3.13 x 10^{-5}	1	3.13 x 10^{-5}	1
	1.56 x 10^{-5}	1	1.56 x 10^{-5}	1	1.56 x 10^{-5}	1
	7.81 x 10^{-6}	1	7.81 x 10^{-6}	1	7.81 x 10^{-6}	1
	3.91 x 10^{-6}	1	3.91 x 10^{-6}	1	3.91 x 10^{-6}	1
	1.95 x 10^{-6}	1	1.95 x 10^{-6}	1	1.95 x 10^{-6}	1
	9.77 x 10^{-7}	1	9.77 x 10^{-7}	1	9.77 x 10^{-7}	1

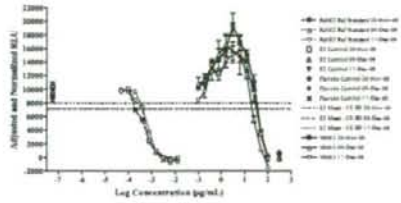
[1] Multiple scores indicate the cell viability scores for concentrations tested in each plate when scores differed from plate to plate. A single cell viability score indicates the score that was given for that concentration on all plates tested.

Phase IIb Antagonist Results for GEN at XDS

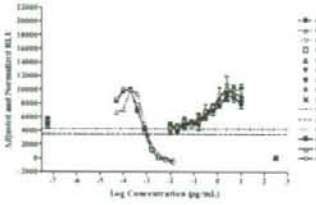


Test concentrations that were cytotoxic (cell viability scores of 2 or greater) were excluded from the graph because they are not used in the determination of antagonist activity.

Phase IIb Antagonist Results for GEN at ECVAM



Phase IIb Antagonist Results for GEN at Hiyoshi



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Interlaboratory Comparison of GEN Antagonist Activity

	Plates Tested	Plates Testing Positive for Antagonism[1]	Plates Testing Negative for Antagonism[1]	CCVAM Meta-data[2]
XDS	3	1	2	4
ECVAM	3	3	0	
Hiyoshi	3	0	3	

[1] Mean adjusted RLU values for a given concentration(s) of test substance is less than the mean minus three times the standard deviation of the plate E2 control values; the test substance is considered positive for antagonist activity - any responses above this threshold is considered negative for antagonist activity.

[2] Indicates that the substance was positive in the single assay in which it was tested.

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Antagonist Comprehensive Testing - Concentrations Tested & Cell Viability for RES

Substance Name	XDS Concentration (log10 M)	XDS Cell Viability[1]	ECVAM Concentration (log10 M)	ECVAM Cell Viability[1]	Hiyoshi Concentration (log10 M)	Hiyoshi Cell Viability[1]
RES	-5.00 x 10^-7	4	1.66 x 10^-7	3	1.66 x 10^-7	1
	8.30 x 10^-7	3.3.3	5.53 x 10^-7	1	5.53 x 10^-7	1
	2.77 x 10^-7	3	2.67 x 10^-7	1	2.63 x 10^-7	1
	1.20 x 10^-7	2.7.1	1.20 x 10^-7	1	1.20 x 10^-7	1
	6.25 x 10^-8	1	6.25 x 10^-8	1	6.25 x 10^-8	1
	3.13 x 10^-8	1	3.13 x 10^-8	1	3.13 x 10^-8	1
	1.56 x 10^-8	1	1.56 x 10^-8	1	1.56 x 10^-8	1
	7.81 x 10^-9	1	7.81 x 10^-9	1	7.81 x 10^-9	1
	3.91 x 10^-9	1	3.91 x 10^-9	1	3.91 x 10^-9	1
	1.95 x 10^-9	1	1.95 x 10^-9	1	1.95 x 10^-9	1
	9.77 x 10^-10	1	9.77 x 10^-10	1	9.77 x 10^-10	1

[1] Multiple scores indicate the cell viability scores for concentrations tested in each plate when scores differed from plate to plate. A single cell viability score indicates the score that was given for that concentration on all plates tested.

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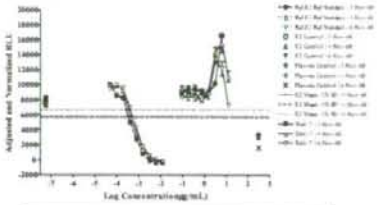
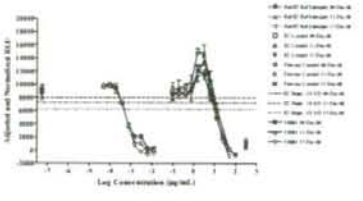
Phase IIb Antagonist Results for RES at XDS



Test concentrations that were cytotoxic (cell viability scores of 2 or greater) were excluded from the graph because they are not used in the determination of antagonist activity.

167

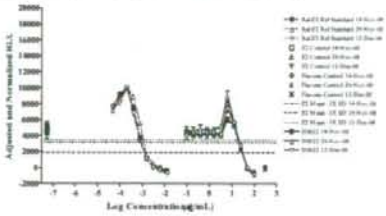
Phase IIb Antagonist Results for RES at ECVAM



Test concentrations that were cytotoxic (cell viability scores of 2 or greater) were excluded from the graph because they are not used in the determination of antagonist activity.

168

Phase IIb Antagonist Results for RES at Hiyoshi



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Interlaboratory Comparison of RES Antagonist Activity

	Plates Tested	Plates Testing Positive for Antagonism[1]	Plates Testing Negative for Antagonism[1]	ICCVAM Meta-data[2]
IDS	3	0	3	+
ECVAM	3	1	0	
Hyasiti	3	2	0	

[1] mean adjusted RLU values for a given concentration(s) of test substance is less than the mean minus three times the standard deviation of the plate EZ control values. the test substance is considered positive for antagonist activity - any responses above the threshold is considered negative for antagonist activity.

[2] + indicates that the substance was positive in the single assay in which it was tested.

Accuracy Assessment of Current LUMI-CELL® ER Assay Validation Phase IIb Test Substance Results and the ICCVAM Meta-Data

Evaluation of Phase IIb Agonist Test Results and the ICCVAM Meta-data (1)

Agonist Test Substance	Laboratory	ER TA Agonist Activity (activities in Bold are discordant from ICCVAM meta-data)
ATZ	ICCVAM Meta-data	Negative
	IDS	Negative (3/3)
	ECVAM	**Positive (3/3)**
	Hyasiti	Negative (2/3)
BBP	ICCVAM Meta-data	Positive
	IDS	Positive (3/3)
	ECVAM	Positive (3/3)
	Hyasiti	Positive (3/3)
DDT	ICCVAM Meta-data	Positive
	IDS	Positive (3/3)
	ECVAM	Positive (3/3)
	Hyasiti	Positive (3/3)
EE	ICCVAM Meta-data	Positive
	IDS	Positive (3/3)
	ECVAM	Positive (3/3)
	Hyasiti	Positive (3/3)

Evaluation of Phase IIb Agonist Test Results and the ICCVAM Meta-data (2)

Agonist Test Substance	Laboratory	ER TA Agonist Activity (activities in Bold are discordant from ICCVAM meta-data)
FLA	ICCVAM Meta-data	Positive
	IDS	Positive (3/3)
	ECVAM	Positive (3/3)
	Hyasiti	Positive (3/3)
GEN	ICCVAM Meta-data	Positive
	IDS	Positive (3/3)
	ECVAM	Positive (3/3)
	Hyasiti	Positive (4/4)
NOR	ICCVAM Meta-data	Positive
	IDS	Positive (3/3)
	ECVAM	Positive (3/3)
	Hyasiti	Positive (3/3)
VIN	ICCVAM Meta-data	Negative
	IDS	Negative (3/3)
	ECVAM	**Positive (3/3)**
	Hyasiti	Negative (3/4)

Evaluation of Phase IIb Antagonist Test Results and the ICCVAM Meta-data (1)

Antagonist Test Substance	Laboratory	ER TA Antagonist Activity (activities in Bold are discordant from ICCVAM meta-data)
API	ICCVAM Meta-data	Positive
	IDS	**Negative (3/3)**
	ECVAM	Positive (2/3)
	Hyasiti	Positive (3/4)
ATZ	ICCVAM Meta-data	Negative
	IDS	Negative (2/3)
	ECVAM	**Positive (3/3)**
	Hyasiti	**Positive (3/3)**
BBP	ICCVAM Meta-data	Negative
	IDS	**Positive (3/3)**
	ECVAM	Negative (2/3)
	Hyasiti	**Positive (3/4)**
CORT	ICCVAM Meta-data	Negative
	IDS	**Positive (3/3)**
	ECVAM	**Positive (3/3)**
	Hyasiti	**Positive (3/3)**

Evaluation of Phase IIb Antagonist Test Results and the ICCVAM Meta-data (2)

Antagonist Test Substance	Laboratory	ER TA Antagonist Activity (activities in Bold are discordant from ICCVAM meta-data)
DDT	ICCVAM Meta-data	Positive
	IDS	**Negative (3/3)**
	ECVAM	Positive (2/3)
	Hyasiti	Positive (2/4)
FLA	ICCVAM Meta-data	Positive
	IDS	Positive (3/3)
	ECVAM	Positive (3/3)
	Hyasiti	**Negative (4/4)**
GEN	ICCVAM Meta-data	Positive
	IDS	**Negative (2/3)**
	ECVAM	Positive (3/3)
	Hyasiti	**Negative (3/3)**
RES	ICCVAM Meta-data	Positive
	IDS	**Negative (3/3)**
	ECVAM	Positive (3/3)
	Hyasiti	Positive (3/3)

Assessment of XDS Agonist Accuracy vs. ICCVAM Meta-Data

		Phase IIb Agonist Classification at XDS		
		Positive	Negative	Total
ICCVAM Agonist Classification	Positive	6	0	6
	Negative	0	2	2
	Total	6	2	8

Concordance = 100% (8/8)
Sensitivity = 100% (6/6)
Specificity = 100% (2/2)
False Negative Rate = 0% (0/6)
False Positive Rate = 0% (0/2)

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Assessment of ECVAM Agonist Accuracy vs. ICCVAM Meta-Data

		Phase IIb Agonist Classification at ECVAM		
		Positive	Negative	Total
ICCVAM Agonist Classification	Positive	6	0	6
	Negative	2	0	2
	Total	8	0	8

Concordance = 75% (6/8)
Sensitivity = 100% (6/60
Specificity = 0% (0/2)
False Negative Rate = 0% (0/6)
False Positive Rate = 100% (2/2)

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Assessment of Hiyoshi Agonist Accuracy vs. ICCVAM Meta-Data

		Phase IIb Agonist Classification at Hiyoshi		
		Positive	Negative	Total
ICCVAM Agonist Classification	Positive	6	0	6
	Negative	0	2	2
	Total	6	2	8

Concordance = 100% (8/8)
Sensitivity = 100% (6/6)
Specificity = 100% (2/2)
False Negative Rate = 0% (0/6)
False Positive Rate = 0% (0/2)

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Assessment of XDS Antagonist Accuracy vs. ICCVAM Meta-Data

		Phase IIb Antagonist Classification at XDS		
		Positive	Negative	Total
ICCVAM Antagonist Classification	Positive	1	4	5
	Negative	2	1	3
	Total	3	5	8

Concordance = 25% (2/8)
Sensitivity = 25% (1/4)
Specificity = 33% (1/3)
False Negative Rate = 80% (4/5)
False Positive Rate = 67% (2/3)

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Assessment of ECVAM Antagonist Accuracy vs. ICCVAM Meta-Data

		Phase IIb Antagonist Classification at ECVAM		
		Positive	Negative	Total
ICCVAM Antagonist Classification	Positive	5	0	5
	Negative	2	1	3
	Total	7	1	8

Concordance = 75% (6/8)
Sensitivity = 100% (5/5)
Specificity = 50% (1/2)
False Negative Rate = 0% (0/5)
False Positive Rate = 67% (2/3)

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Assessment of Hiyoshi Antagonist Accuracy vs. ICCVAM Meta-Data

		Phase IIb Antagonist Classification at Hiyoshi		
		Positive	Negative	Total
ICCVAM Antagonist Classification	Positive	3	2	5
	Negative	3	0	3
	Total	6	2	8

Concordance = 38% (3/8)
Sensitivity = 60% (3/5)
Specificity = 0% (0/3)
False Negative Rate = 10% (2/5)
False Positive Rate = 100% (3/3)

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Assessment of Agonist Experiment by Experiment Accuracy vs. ICCVAM Meta-Data

Phase IIb Agonist Classification on an Experiment by Experiment Basis Across Participating Laboratories		ICCVAM Agonist Classification		
		Positive	Negative	Total
	Positive	56	0	56
	Negative	8	11	19
	Total	64	11	75

Concordance = 89% (67/75)
Sensitivity = 100% (56/56)
Specificity = 58% (11/19)
False Negative Rate = 0% (0/56)
False Positive Rate = 42% (8/19)

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Assessment of Antagonist Experiment by Experiment Accuracy vs. ICCVAM Meta-Data

Phase IIb Antagonist Classification on an Experiment by Experiment Basis Across Participating Laboratories		ICCVAM Antagonist Classification		
		Positive	Negative	Total
	Positive	32	27	59
	Negative	17	2	19
	Total	49	29	78

Concordance = 44% (34/78)
Sensitivity = 54% (32/59)
Specificity = 11% (2/19)
False Negative Rate = 46% (27/59)
False Positive Rate = 89% (17/19)

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Evaluation of LUMI-CELL® ER Phase IIb Test Failure Rates

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Agonist and Antagonist Acceptance Criteria and Failure Rates (1)

- NICEATM calculated the failure rates for the test plates from the comprehensive testing of the eight coded agonist and eight coded antagonist substances used in this phase. The percentages of agonist and antagonist test plates that failed acceptance criteria across the three participating laboratories were 16% (7/45) and 14% (6/44), respectively.
  - At Hiyoshi Corporation, 19% (3/16) of agonist test plates failed acceptance criteria but all antagonist test plates (14) passed acceptance criteria
  - At XDS, Inc., all agonist (13) and antagonist (12) test plates passed acceptance criteria
  - At the ECVAM laboratory, 25% (4/16) of agonist test plates and 33% (6/18) of antagonist test plates failed acceptance criteria

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Agonist Test Plate Failure Rates using Current Acceptance Criteria

Laboratory	Total Number of Plates Tested	Number of Plates Passing Acceptance Criteria	Number of Plates Failing Acceptance Criteria	Failed DMSO Only[1]	Failed Induction Only[2]	Failed both DMSO and Induction
XDS	13	13	0	-	-	-
ECVAM	16	12	4	-	1	3
Hiyoshi	16	13	3	1	1	1
Overall	45	38	7	1	2	4

[1]DMSO = averaged DMSO control RLU value must be within 2.5 times the standard deviation (SD) of the historical DMSO control value.
[2]Induction = Maximum-fold induction

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Antagonist Test Plate Failure Rates using Current Acceptance Criteria

Laboratory	Total Number of Plates - Tested	Number of Plates Passing Acceptance Criteria	Number of Plates Failing Acceptance Criteria	Failed DMSO[1]	Failed Reference Standard Curve[2]
XDS	12	12	0	-	-
ECVAM	18	12	6	4	2
Hiyoshi	14	14	0	-	-
Overall	44	38	6	4	2

[1]DMSO = averaged DMSO control RLU value must be within 2.5 times the standard deviation (SD) of the historical DMSO control value.
[2]The Rat/E2 reference standard concentration response curve should be sigmoidal in shape and have at least three values within the linear portion of the concentration response curve.

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Summary of Phase IIb of the LUMI-CELL® ER Assay International Validation Study

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Summary (1)

- Eight coded test substances covering a range of ER agonist activities were tested in at least three independent experiments at each of the participating laboratories
  - BBP, DDT, EE, FLA, GEN, and NON were reproducibly classified as estrogenic agonists in all of the participating laboratories
  - ATZ tested negative for estrogenic activity at XDS (2/3) and Hiyoshi (2/3), but was positive at ECVAM (3/3)
  - VIN tested negative for estrogenic activity at XDS (3/3) and Hiyoshi (3/4), but was positive at ECVAM (3/3)

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Summary (2)

- Eight coded test substances covering a range of ER antagonist activities were tested in at least three independent experiments at each of the participating laboratories
- Classification of test substances as positive or negative for antagonism varied across the laboratories.

	Test Substance Antagonist Classification		
Test Substance	XDS	ECVAM	Hiyoshi
API	-	+	+
ATZ	-	+	+
BBP	+	-	+
CORT	+	+	+
DDT	-	+	+
FLA	-	+	-
GEN	-	+	-
RES	-	+	+

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Summary (3)

- Agonist and Antagonist reference standards and controls were evaluated for intra- and inter-laboratory reproducibility
  - Reference standard and control data were reproducible within laboratories
  - Statistically significant differences were observed in intra- and inter-laboratory reference standard and control values.
  - It was not possible to identify the causes for these differences but some of the contributing factors may be:
    - Lot-to-lot differences in cell culture media and tissue culture supplies (for intra- and inter-lab differences)
    - Differences in luminometers (for inter-lab differences)
  - This underscores the importance of developing and maintaining an historical control database for each individual laboratory

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Progression to Phases III and IV of the LUMI-CELL® ER Assay International Validation Study

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Recommendation to the SMT

- To initiate Phases III and IV using current protocols

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Historical Database Values Established for Acceptance Criteria for Phases III and IV

	Units	Mean	SD	Mean Plus 2.0 Times SD	Mean Minus 2.5 Times SD
			XDS[1]		
DMSO	RLU	2157	3012	7186	0
E2	Adjusted RLU	5360	737	10212	6519
			ECVAM		
DMSO	RLU	3665	1420	7228	102
E2	Adjusted RLU	9131	736	11032	7240
			Hypaln		
DMSO	RLU	5130	1663	8337	923
E2	Adjusted RLU	9805	805	7756	3442

Abbreviations: DMSO = Dimethyl sulfoxide; RLU = Relative Light Units; SD = Standard Deviation
[1]Only XDS will be performed in Phase IV testing. Phase IV testing involves the testing of substances that are included on the ICCVAM list of Reference Substances for the Validation of ER Binding and Transcriptional Activation Assays, but that are not included in the subset of 53 test substances on the ER minimum list.
[2]DMSO values can not be below zero.

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

平成20年度分担研究報告書

OECD活動と国際協調

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研究要旨

2008年10月にベルリンで開催された　皮膚刺激性専門家会議に参加し、新たなテストガイドライン案について意見交換するとともに、日本のバリデーション結果を報告した。

2008年11月にイスプラで開催されたOECD/ Endocrine Disruption Testing and Assessment Task Force (EDTA) Validation Management Team-Non Animal (VMT-NA)会議に参加し、現在進めている国際バリデーション研究の進捗について意見交換した。

皮膚感作性試験 Local Lymph Node Assay (LLNA)の変法である非放射線物質による LLNA法および培養表皮モデルLabCyte　EPI-MODEL24を用いた皮膚刺激性試験のOECDガイドライン化を目指し、Standard　Project　Submission　Form (SPSF) を2009年1月までに厚生労働省のナショナルコーディネーターを通してOECDに提出した。

A. 研究目的

動物実験代替法に関しては、化粧品の安全性評価法を中心に、多くの検討が行われている。皮膚腐食性試験や光毒性試験代替法などにおいては、欧米および我が国において大規模なバリデーション研究と専門家による第三者評価（以下、第三者評価と記す）が実施され、一部がOECDのガイドラインに取り入れられ、化学物質の評価にも用いられている。しかし、感作性試験や生殖毒性試験など、まだ開発やOECD基準に則ったバリデーション研究がなされていないものも多い。一方、内分泌かく乱化学物質の*in vitro*評価法については無細胞系受容体結合試験、酵母等各種導入受容体結合試験、各種受容体導入レポーター遺伝子転写活性化試験（Lumi-cell法など）、化学物質評価研究機構（CERI）が開発したHeLa細胞をベースにしたエストロジェン受容体αに対するレポーターアッセイ試験法の他、アロマターゼ活性化試験など、いくつかの方法が開発され、OECD基準に則ったバリデーション研究が行われている。DNA損傷性を調べるコメットアッセイについても、*in vitro*および*in vivo*の試験法が開発されているが、データの評価、解釈のみならず方法論に関しても未熟であり、国際的なガイドラインは作成されていない。

本研究班はこれら今まで評価が遅れていた化学物質の安全性評価のための試験法をOECDの基準に則ってバリデーション研究と第三者評価を行うものである。また、極めて多大な労力を有し、大学や個々の研究機関、更には、一国では実施困難な多施設バリデーション研究と第三者評価を国際的な協力のもとで実施し、本研究で検討した試験方法のOECDガイドライン化を目指すものである。そこで、我々の開発した方法を将来、OECDの試験法ガイドライン化するための活動を行うとともに、関連するOECD活動に協力した。

本報告書では、日本で開発あるいは日本が中心となって開発している方法を中心に、OECDにおける活動および国際協調をまとめた。

B. 研究方法および結果

B-1 皮膚刺激性専門家会議

2008年10月21日～22日にベルリン BfR (Federal Institute for Risk Assessment)で開催された皮膚刺激性専門家会議に小島 肇（国立医薬品食品衛生研究所）が参加した。

EUより提案のあった培養表皮モデルを用いた皮膚刺激性試験テストガイドライン案について意見交換するとともに、培養表皮モデル LabCYte EPI-MODEL24 を用いた皮膚刺激性試験に関する日本のバリデーション結果を報告し、その内容を今後考慮するように求めた。

添付資料1および2参照。

B-2 OECD/ Endocrine Disruption Testing and Assessment Task Force (EDTA) Validation Management Team-Non Animal (VMT-NA)での会合

2008年11月19日～21日にフランス パリOECD本部で開催されたOECD/EDTA VMT-NAに日本から小野 敦博士、小島 肇（以上、国立医薬品食品衛生研究所）、武吉正博博士（化学物質評価研究機構）が出席した。OECD/EDTA VMT-NAで検討が進められている各種の内分泌かく乱物質スクリーニングの進捗について報告があり、各国の代表とその内容について意見交換した。

我々は、日本で進捗中のバリデーション研究内容について報告した。特に、日本で開発されたHeLa細胞をベースにしたエストロジェン受容体αに対するレポーターアッセイについては、OECDガイドライン成立を目指し、日本主導でバリデーションの準備を進めていると報告し、その内容に助言を得た。

添付資料3および4参照。

B-3 眼刺激性試験専門家会議

2008年12月4日～5日にワシントンD.C.で開催された眼刺激性専門家会議には日本から参加しなかった。

しかし、米国より提案のあった摘出牛角膜試験および摘出鶏眼球試験テストガイドライン案について事前に意見を送った。

B-4 SPSFの作成

皮膚感作性試験Local Lymph Node Assay (LLNA)の変法である非放射線物質によるLLNAおよび培養表皮モデルLabCyte EPI-MODEL24を用いた皮膚刺激性試験のOECDガイドライン化を目指し、試験法の予備登録である Standard Project Submission Form (SPSF)を2009年1月までに厚生労働省のナショナルコーディネーターを通してOECDに提出した。

添付資料5、6および7参照。

C. 考察

本研究班のテーマである「化学物質リスク評価法の国際的バリデーション」の目的は、安全性評価に有用な新規試験法を公定化することである。その最終的な目標がOECDガイドラインの確立であることから、これを目指してSPSFを提出し、本研究班のバリデーション結果から国際的検討に至るよう努力するものである。

なお、OECDガイドラインとして完成させるまでには、短くても3年、通常5年以上掛かることを銘記しなければならない。この点でより迅速な国際合意を図るために共同研究は重要と考えている。

E. 結論

OECDの新たなガイドラインの成立に協力するとともに、日本からもSPSFを提出して積極的な試験法の開発を進めている。

F. 健康危険情報
なし

G. 知的財産権の出願・登録状況
なし

H. 研究発表
1．論文発表
1) 小島肇夫：in vivo 経皮吸収試験法、最新・経皮吸収剤～開発と基礎から申請のポイントまで～、株式会社情報機構、東京、pp. 95-103 (2008)
2) 小島肇夫：in vitro 経皮吸収試験法、最新・経皮吸収剤～開発と基礎から申請のポイントまで～、株式会社情報機構、東京、pp. 104-113 (2008)
3) 小島肇夫：安全性評価と動物実験代替法の現状、薬学雑誌、128 (5) 747-752. (2008)
4) 小島肇夫：動物実験の 3 Rs における国内外の動向、ファルマシア、44(9)、857-861 (2008)
5) 小島肇夫:REACH 対応に必要な動物実験代替法の現状、コスメティックステージ、2 (5)、1-4 (2008)
6) 小島肇夫：動物実験代替法に関する 2008 年の国際動向、Fragrance Journal、2009-1、65-69 (2009)
7) 小島肇夫：動物実験代替法の現状と展望、J. Environ Dermatol Cutan Allergol. 3 (1)、1-6 (2009)
8) Omori. T., Idehara. K., Kojima H., Sozu. T., Arima. K., Goto. H., Hanada. T., Ikarashi. Y., Inoda. T., Kanazawa. Y., Kosaka. T., Maki. E., Morimoto. T., Shinoda. S., Shinoda. N., Takeyoshi. M., Tanaka. M., Uratani. M., Usami. M., Yamanaka. A., Yoneda. T., Yoshimura. I., Yuasa. A. : Interlaboratory validation of the modified murine local lymph node assay based on adenosine triphosphate measurement. Journal of Pharmacological and Toxicological Methods 58 11-26 (2008)
9) Arai. S., Yamamoto. N., Kato M., and Kojima. H.: An *in vitro* evaluation methods to test ocular irriation using a human corneal epithelium model. *Altern. Animal Test. EXperiment*. 13 (2). 83-90 (2008)
10) 小島肇夫：動物実験の 3 Rsにおける国内外の動向、城西大学生命科学研究センター報告第7号、p37-50 (2009)

2．学会発表
1) Kojima. H.: Report JaCVAM. ECVAM Scientific Advisory Committee. Ispra (May. 2008)
2) 小島肇夫:REACH 対応と動物実験代替法、第 128 回 FJ セミナー、東京 (2008)
3) Kojima. H.: Japanese Collaboration on Alternative to Animal Toxicology Testing. World Congress on in Vitro Biology. Tucson (2008)
4) Kojima. H.: JaCVAM Update. Scientific Advisory Committee on Alternative Toxicological Methods. Bethesda. (June. 2008)
5) 小島肇、武吉正博、大森崇、寒水孝司、有馬和範、出原賢治、金澤由基子、牧栄二、中桐直人、五十嵐良明、田中正志、吉村功、湯浅敦子：LLNA-BrdU 法の施設間バリデーション研究、第 35 回日本トキシコロジー学会学術年会、東京 (2008)
6) 小島肇、武吉正博、出原賢治：非 RI 法による皮膚感作性試験代替法(LLNA 法)のバリデーション研究－試験法概要－、第 15 回日本免疫毒性学会学術大会、東京 (2008)
7) 出原賢治、小島肇、武吉正博：非 RI 法による LLNA 法の比較、第 38 回日本皮膚アレルギー・接触皮膚炎学会総会学術大会、大阪 (2008)
8) Kojima. H.: Report JaCVAM. ECVAM Scientific Advisory Committee. Brussels (November. 2008)
9) Kojima. H.: International Current of 3Rs International Symposium on the 3Rs Promotion in Asia. Saitama (2008)
10) 小島 肇：医薬部外品の製造販売承認申請における安全性に関する資料のあり方検討会報告、日本動物実験代替法学会第 21 回大会、埼玉 (2008)
11) 杉山真理子、河合敬一、小島 肇、寒水孝司、辰見寿、夏秋優、森福義：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あり方検討委員会報告 皮膚刺激性分科会からの報告、日本動物実験代替法学会第 21 回大会、埼玉 (2008)
12) 金澤由基子、横関博雄、中田土起丈、坂口斉、大野泰雄、小島 肇：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あり方検討委員会報告 感作性試験分科会からの報告、日本動物実験代替法学会第21回大会、埼玉 (2008)
13) 瀬戸 洋一、萩野 滋延、畠 賢一郎、森田正道、平野 耕治、金子 豊蔵、小島 肇：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あり方検討委員会報告 眼刺激性分科会からの報告、日本動物実験代替法学会第 21 回大会、埼玉 (2008)
14) 藤井まき子、小島 肇、杉林堅次、上月裕一、桑原裕史：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あり方検討委員会報告 皮膚透過・経皮吸収試験分科会からの報告、日本動物実験代替法学会第 21 回大会、埼玉 (2008)
15) 笠松俊夫、江幡真也、林 真、能美建彦、本間正充、小島 肇：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あり方検討委員会報告 遺伝毒性分科会からの報告、日本動物実験代替法学会第 21 回大会、埼玉 (2008)

16) 武吉正博、小島　肇、大森崇、寒水孝司、有馬和範、出原賢治、金澤由基子、牧栄二、中桐直人、五十嵐良明、田中正志、吉村功、湯浅敦子：LLNA-BrdU 法の施設間バリデーション研究、日本動物実験代替法学会第 21 回大会、埼玉（2008）

17) 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　予備試験結果、日本動物実験代替法学会第 21 回大会、埼玉（2008）

18) 小島　肇：in vitro 遺伝毒性試験－光と影－、第 37 回日本環境変異原学会、沖縄（2008）

19) 小島　肇：毒性試験における培養細胞の利用、安全性評価研究会２００８年冬のセミナー、東京（2008）

20) 小島　肇：動物実験適正化のグローバルな動き－代替法の動きを中心に－、日本制約工業協会医薬品評価委員会　第 102 回基礎研究部会総会、京都（2009）

21) Kojima, H., Current aspects of LLNA-DA and LLNA-BrdU as alternatives for skin sensitizer classification in Japan, 2009 Winter Conference of Korean Society of Alternatives to Animal Experiments, Seoul (February, 2009)

22) Kojima, H., Report JaCVAM, ECVAM Scientific Advisory Committee, Ispra (March, 2009)

23) Tice, RR., Deal, F., Ceger, P., Allen, D., Gordon, J ., DeLange, J., Bremer, S., Nakamura, M., Kojima, H., Ono, A., Stokes, W.: Establishing a Historical Database for a Multi-phased International Validation Study of a Stably-Transfected Estrogen Receptor (ER) Transcriptional Activation (TA) Test Method, 48th Annual SOT meeting, Baltimore (2008)

24) Strickland, J., Paris, M., Allen, D., Tice, RR., Kojima, H., Prieto, P., Wind, M., and Stokes, W. : ICCVAM/NICEATM/ECVAM/JaCVAM Scientific Workshop on Acute Chemical Safety Testing: Advancing *In Vitro* Approaches and Humane Endpoints for Acute Systemic Toxicity Evaluations, 48th Annual SOT meeting, Baltimore (2008)

I. 添付資料

資料 1：DRAFT REPORT OF THE OECD EXPERT CONSULTATION MEETING FOR THE REVISION OF THE DRAFT *IN VITRO* SKIN IRRITATION TEST GUIDELINE

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(REV 1) Paris 13 November 2008
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DRAFT REPORT OF THE OECD EXPERT CONSULTATION MEETING FOR THE REVISION OF THE DRAFT *IN VITRO* SKIN IRRITATION TEST GUIDELINE

20-21 October, 2008, Federal Institute for Risk Assessment (BfR), Berlin-Marienfelde, Germany

INTRODUCTION

1. The Secretariat opened the meeting and OECD acknowledged the Federal Institute for Risk Assessment (BfR) for once again hosting an expert meeting and introduced Professor Horst Spielmann, who gave a presentation on the history and activities of the BfR. Valérie Zuang of the European Commission (EC has the project lead) introduced the topic and gave a presentation on the background to the ECVAM Skin Irritation Validation Study (SIVS).

2. The Secretariat welcomed participants and described OECD procedures and the work of the Test Guidelines Programme and emphasized that participants ideally are present as experts, independent of any national position. It was also emphasised that due to the animal testing and marketing bans coming into force in EU in March 2009 as imposed by the 7th Amendment to the Cosmetics Directive, there was a real time constraint to be able to meet this deadline for the EC. The Secretariat explained that there probably have to be two parallel processes, one for the EU and one for the OECD, for the Test Guideline developmental process. The Secretariat introduced Horst Spielmann as the acting co-chair together with the Secretariat, which was approved by the meeting.

3. The draft Agenda was slightly revised to also accommodate presentations by Elke Genschow (BfR) and Hajime Kojima (MHLW JaCVAM), see Annex 1 for an agenda.

4. Experts introduced themselves to the meeting (for a list of participants see Annex 2).

5. Karen Hamernick (US EPA) explained that the US had considerable problems with adequately reviewing the proposed draft Test Guideline and the presented analyses and data, including the validation studies due to the limited time given prior to the meeting. She further explained that the US will not endorse or make any concluding decisions on the TG during the meeting and a primary comment was that the TG does not meet US regulatory needs for a number of applications. She will listen and take the information from the meeting back to relevant stakeholders in the US. The Secretariat concurred that the time to review the documents related to the validation of the modified EpiDerm™ assay and the similar SkinEthic RHE™ assay was extremely short, since these validation documents were not available until a few days before the meeting due to the fact that they still were in the ESAC peer review process. It should also be emphasised that the recalculation results of the classifications of the chemicals in the SIVS validation study (when the cut-off value was changed from 2.0 to 2.3), as were presented by Elke Genshow at the meeting where not available for review prior to the meeting. The strategy from the Secretariat is that usually the supporting documentation should be available to experts for their review 4-6 weeks before a meeting, but at this special occasion that deadline was not possible to meet.

PRESENTATIONS AND DISCUSSIONS ON THE PERFORMANCE OF THE THREE TESTS

6. Valérie Zuang noted prior to her presentation that the draft Test Guideline submitted to the OECD, together with the SPSF and accompanying documents in January 2008, was based on the results of the

ECVAM Skin Irritation Validation Study. She gave a presentation on the "*Outcome of the ECVAM Validation Study on In Vitro Tests for Acute Skin Irritation.*" The outcome of the study was reviewed by ESAC (ECVAM Scientific Advisory Committee) that issued a statement of scientific validity in April 2007 saying that: *...the EPISKIN™ method showed evidence of being a reliable and relevant stand-alone test for predicting rabbit skin irritation, when the endpoint is evaluated by MTT reduction, and for being used as a replacement for the Draize Skin Irritation Test (OECD TG 404 & Method B.4 of Annex V to Directive 67/548/EEC) for the purposes of distinguishing between R38 skin irritating and no-label (non-skin irritating) test substances. At the present time the IL-1α endpoint was regarded as a useful adjunct to the MTT assay, as it has the potential to increase the sensitivity of the test, without reducing its specificity. This endpoint could be used to confirm negatives obtained with the MTT endpoint. At this time, due to its high specificity, the EpiDerm model reliably identifies skin irritants, but negative results may require further testing (e.g. according to the tiered strategy, as described in the OECD TG 404). Improvement of the EpiDerm protocol should be made to increase the level of sensitivity...."*

7. She also explained that the reason for inclusion of the interleukin 1 alpha (IL-1α) release endpoint in the SIVS was to check R38-classified borderline chemicals (which were the ones that were mostly mis-classified). The prediction model for IL-1α in her presentation had been developed post-hoc. Inclusion of the IL-1α endpoint in the EpiSkin™ protocol considerably increased the sensitivity of the test without significantly decreasing its specificity. For EpiDerm™ no improvement to the outcome was obtained. It is important to note however, that the IL-1α endpoint did not show adequate between-laboratory reproducibility. The meeting reconfirmed the previous decision during the telephone conference call 2 weeks before the meeting, not to include the IL-1α endpoint in the draft Test Guideline, because it is not regarded sufficiently validated.

8. Manfred Liebsch (BfR) presented the SIVS follow-up study in four laboratories with an updated EpiDerm™ Skin Irritataion Test (SIT) protocol and the supporting documents. He especially mentioned Meeting document # 5.1, "Test submission template", as a core document. While basic elements of the protocol (dose and post-incubation period) were kept unchanged, according to the improved barrier function of the EpiDerm™ model, the chemical exposure time had to be increased to 60 minutes to achieve the required sensitivity. Of the 60 minutes, for technical reasons, 25 minutes were performed at room temperature, and 35 minutes were performed at 37°C / 5% CO_2 in the incubator. While the latter change (37° C incubation) did not change the predictive performance, it reduced the data variability. Fiftynine chemicals were tested with this protocol in phase 1 at MatTek, and the 20 reference chemicals were then tested blind in three experienced and one naïve laboratory. In summary, based on the major call across laboratories, the two referenece chemicals classified false positive in the validated reference test (EPISKIN™) were confirmed false positive in EpiDerm™, while of the three chemicals classified false negative in the validated reference test, two were confirmed false negative, and one (#12, terpenylacetate) was correctly identified in all four laboratories as irritant chemical. Thus, a sensitivity and specificity of both 80% was achieved and the reqirement to be equal or better than the validated reference test) was met. Regarding the IL-1α endpoint, it was tested with the 20 chemicals for the optimised EpiDerm™, and it gave no contribution to the performance of the test method.

9. It should be noted that both the validation document packages (CORRELATE Submission Package) for the modified EpiDerm™ and SkinEthic RHE™ were kindly made available to the meeting by ECVAM. This is usually not possible prior to the finalisation of the ESAC peer review process and the issue of an ESAC statement. However, since a decision by the peer review panel had already been taken, there was no real constraints not to make the documents available to the meeting.

10. Manfred Liebsch pointed out that EpiDerm™, EpiSkin™ and SkinEthic RHE™ all used basically the same protocol during the validation, with only minor deviations. The purpose of the EpiDerm™ follow-up validation study was to increase the test sensitivity by an increase of the exposure time from 15

minutes to 60 minutes..Likewise, an exposure period of 42 minutes was optimal for the SkinEthic model to achieve results comparable with the validated reference EPISKIN. Horst Spielmann concluded that only the exposure volumes and exposure times needed to be optimised for each individual epidermis model according to the barrier function, while the basic elements, a of the method, a post-incubation periopd of 42 hrs and the prediction model were identical for all reconstructed epidermis models.

11. Since several chemicals are irritant on rabbit only (Meeting document #6 and presentation slide 8), a discussion followed on the use of human skin patch data and the usability of this data. Horst Spielmann mentioned that the rabbit data is very conservative and over-predict many chemicals compared to human patch data. However, since the predictive 4hr human patch test failed to be adopted as an OECD Test Guideline in 1996 for legislative reasons even the scientific discussions of the human patch data is not easy.

12. A discussion on the usability of the proposed Test Guideline and whether it could be applied by US agencies was initiated by Karen Hamernick. In the US, corrosives and irritants are always tested together in the rabbit test and the skin corrosion Test Guidelines 430, 431 and 435 are only used for positive screening by US agencies. Manfred Liebsch explained the tiered testing strategy outlined in UN GHS, which is also attached to TG 403/404, where you may go from validated in vitro tests to final testing in the rabbit, if necessary. According to Karen Hamernick, the UN GHS system has not been implemented in the US. Karen further wondered if the model could be used for both irritation and corrosion testing. Manfred Liebsch explained that that, although the protocols of the skin corrosion test (SCT) and the skin irritation test (SIT) are both employing MTT as endpoint, they are entirely different: while the MTT assay performed immediately after short term chemical exposure is specific for corrosive effects, only substances predicted "non corrosive" in the SCT should be tested in the SIT to discriminate irriants from non-irritants. Of course all corrosives are also positive in the SIT, and a discrimination of corrosives and irritants would not be possible if only the SIT is performed.

13. Karen Hamernick also raised a concern regarding false negatives obtained with the *in vitro* corrosivity assays during the validation studies. Joao Barroso replied that most of these were either direct MTT reducers, or classified corrosives according to pH class under the testing strategy appended to OECD TG 404.

14. Nathalie Alepée (L'Oréal) presented the SkinEthic RHE™ validation study and explained that only exposure time (42 minutes) is different from the other two tests. In phase 1, 20 chemicals from the SIVS were tested in three laboratories with very good reproducibility. In phase 2, the same 20 chemicals (now coded) were tested in the same three laboratories with equally good reproducibility and a very good predictive performance: 90% sensitivity and 80 specificity were obtained, both in single laboratories and across all laboratories. The IL-1α endpoint did not contribute to the performance. The optimisation to 42 minutes exposure time was done with another set of chemicals before the 20 were tested.

15. Elke Genshow (BfR) presented for the studies preceeding the SIVS and those following the SIVS a re-calculation of the classification data obtained according to the change of the EU GHS cut-off value from 2.0 to 2.3 for GHS category 2. The EU will adopt only category 2 of the GHS and drop the category for weak irritants (category 3). Expectedly, the general trend of the data for all skin models was that sensitivity goes up, specificity goes down and the accuracy goes down slightly. Since the document provided in advance to the ECM suffered a bit from the fact that also studies preceeding the SIVS were called "optimisation studies", the document will be restructured and calculations from ECVAM added, and provided as a joint BfR-ECVAM document for the envisaged expert consultation in the USA (see para 15).

16. Following a new discussion on the usability of the Test Guideline for US agencies, the Secretariat suggested that in order to have an appropriate review of the existing data and the regulatory needs of OECD member countries, an Expert meeting could be held in US sometime early 2009. That would give member countries time to properly review the proposal and discuss the data and the draft Test Guideline in

more detail. The Secretariat will consult with the US after the meeting for arrangements of a meeting preferably sometime in late February to late March 2009.

17. Hajime Kojima (JaCVAM) gave a presentation on Japanese in vitro skin irritation assays. A number of skin models like EpiDerm™, and others are readily available in Japan and several are under development or validation. The LabCyte™: epidermal skin model is under validation with the 20 reference chemicals from the ECVAM Performance Standards document, with the exemption of no 13, which is not available in Japan. The validation study is scheduled to end in December 2008 and the peer review will be coordinated by JaCVAM and will be initiated in the 2nd quarter of 2009.

REVISION OF THE DRAFT TEST GUIDELINE

18. The meeting participants were asked to go through the recent comments received from the EC, US, Sweden and Denmark and make the appropriate changes to the draft Test Guideline. Karen emphasised that before a paragraph by paragraph revision is started, a sentence should be added as a footnote to the first page of the draft TG to clarify the US Position, stating that: "*The US can not endorse a draft test guideline or annexes at this time and will need additional time to review any supporting materials and any proposals for its content, language, and utility with appropriate stakeholders*".

19. Juan Riego Sintes (EC) explained the Commission deadlines. To be able to circulate the draft TG to the EU national coordinators for approval, he needs to circulate the draft TG within days after the meeting. The expected submission to the inter-commission services should be accomplished by mid/late November, this to meet the deadline of the re-election and closing of the EP by March 2009.

20. The meeting participants started going through the draft in a paragraph-wise manner making changes where appropriate, as suggested by the EC (for a clean version of the draft Test Guideline please see annex 3).

21. A crucial point of discussion was again the exact language in the first ten paragraphs of the draft TG regarding the proposed use of the TG and it was agreed that the text should include that the TG could be used as a stand alone *in vitro* test replacing the *in vivo* rabbit test, as a screen, or as a part of a sequential testing strategy in a weight-of-evidence approach. It was also agreed that the text should state that regulatory requirements and needs in member countries will decide the exact role (stand alsone sceen or part of a tiered testing strategy) of this TG.

22. Another issue that was extensively discussed was the classification of chemicals in accordance with UN GHS category 2. It was agreed that the TG should mirror that it only provide means for classification of skin irritation GHS sub-category 2, but there should be no restrictions for member countries to do additional testing if they require also for the classification of sub-category 3.

23. On the question of testing of constructs from a wide range of genetic backgrounds for the issue of idiosyncratic populations, the meeting agreed this was out of the scope of the TG.

24. Performance standards developed for the SIVS should be clearly reflected in the annex 2 of the draft TG. This section was considerably revised since information was missing from meeting document No. 4.4. It was evident that three chemicals will be reclassified and this will result in a less balanced set of reference chemicals from 10/10 (classified cat.2/non-classified) to 7/13. The meeting agreed that 3 new classified irritants should be added and 3 old non-classified chemicals to be taken out. This would also result in new predictive values. In addition the list of proficiency chemicals will have to be decided.

FOLLOW-UP