

STUDY TITLE

HR-001: DNA Repair Test (Rec-Assay)

DATA REQUIREMENT

Required under U.S. EPA FIFRA Guidelines, Subdivision F

AUTHOR

[REDACTED]

STUDY COMPLETED ON

March 14, 1995

PERFORMING LABORATORY

The Institute of Environmental Toxicology
Suzuki-cho 2-772, Kodaira-shi, Tokyo 187, Japan

LABORATORY PROJECT ID

IET 94-0141

SPONSOR

Sankyo Co., Ltd.
7-12, Ginza 2-chome, Chuo-ku,
Tokyo 104, Japan

STATEMENT OF DATA CONFIDENTIALITY CLAIMS

HR-001: DNA Repair Test (Rec-Assay)
(IET 94-0141)

This report contains the unpublished results of research sponsored by Sankyo Co., Ltd. These results may not be published, either wholly or in part, or reviewed or quoted in any other publication without the authorization of Sankyo Co., Ltd.

This document is not the property of EFSA and is provided for giving full effect to the right of public access to documents under EU law.
The document may be subject to rights such as intellectual property and copy rights of third parties.
Furthermore, this document may fall under a regulatory data protection regime.
Consequently, any publication, distribution, reproduction and/or publishing and any commercial exploitation and use of this document may therefore be prohibited and violate the rights of its owner.

GLP STATEMENT

HR-001: DNA Repair Test (Rec-Assay)
(IET 94-0141)

This study was conducted in conformity to Good Laboratory Practice standards (GLPs) of MAFF in Japan (59 NohSan No. 3850, 1984), EPA in U.S.A. (FIFRA: 40 CFR 160, 1989), and OECD (OECD Principles of GLP, 1981).

The Institute of Environmental Toxicology

Administrator:

[Redacted Signature]

Director General

Mar. 14, 1995

Date

Study Director:

[Redacted Signature]

Senior Scientist
Laboratory of Genetic Toxicology
Toxicology Division

March 14, 1995

Date

Sponsor:

Sankyo Co., Ltd.

Date

Submitter:

Date

FLAGGING STATEMENT

HR-001: DNA Repair Test (Rec-Assay)
(IET 94-0141)

This page is intentionally left blank for country specific requirements.

This document is not the property of EFSA and is provided for giving full effect to the right of public access to documents under EU law.
The document may be subject to rights such as intellectual property and copy rights of third parties.
Furthermore, this document may fall under a regulatory data protection regime.
Consequently, any publication, distribution, reproduction and/or publishing and any commercial exploitation and use of this document may therefore be prohibited and violate the rights of its owner.

HR-001: DNA Repair Test (Rec-Assay)
(IET 94-0141)

OBJECTIVE

The purpose of this study was to evaluate the DNA-damaging potential of HR-001 in bacteria.

SPONSOR

Name: Sankyo Co., LTD.
Address: 7-12, Ginza 2-chome, Chuo-ku, Tokyo 104, Japan

TESTING INSTITUTION

Name: The Institute of Environmental Toxicology
Address: Suzuki-cho 2-772, Kodaira, Tokyo 187, Japan

TESTING FACILITY

Name: Kodaira Laboratories
The Institute of Environmental Toxicology
Address: Suzuki-cho 2-772, Kodaira, Tokyo 187, Japan
Administrator: XXXXXXXXXX
Director General

HR-001: DNA Repair Test (Rec-Assay)
(IET 94-0141)

STUDY PERIOD

Establishment of contract:	January 12, 1995
Approval of protocol:	February 7, 1995
Testing period:	
Initiation of experiment:	February 14, 1995
Termination of experiment:	February 15, 1995
Draft report preparation:	February 23, 1995
Comments from sponsor:	March 13, 1995
Final report preparation:	March 14, 1995

STORAGE OF RECORDS

All records obtained during the conduct of this study will be retained in the archive of this institution for ten years after the submission of the final report to the sponsor. Storage after this period will be negotiated between the Institute of Environmental Toxicology and the sponsor.

HR-001: DNA Repair Test (Rec-Assay)
(IET 94-0141)

STUDY DIRECTOR AND SUPERVISORY PERSONNEL

We, the undersigned, hereby declare that the study was performed under our supervision in conformity to the GLPs of MAFF in Japan (59 NohSan No. 3850, 1984), EPA in U.S.A. (FIFRA: 40 CFR 160, 1989), and OECD (OECD Principles of GLP, 1981) and the Guidelines of MAFF in Japan (59 NohSan No. 4200, 1985) and EPA in U.S.A. (Pesticide Assessment Guidelines, Subdivision F, 1991).

Study Director:

[Redacted Signature]

March 14, 1995
Date

Senior Scientist
Laboratory of Genetic Toxicology
Toxicology Division

Mutagenicity:

[Redacted Signature]

March 14, 1995
Date

Acting Chief
Laboratory of Genetic Toxicology
Toxicology Division

Executive Supervisor:

[Redacted Signature]

March 14, 1995
Date

Director of Toxicology

HR-001: DNA Repair Test (Rec-Assay)
(IET 94-0141)

PERSONNEL IN CHARGE

Mutagenicity Examination:

[REDACTED] D.V.M., Ph.D.
Senior Scientist, Laboratory of Genetic Toxicology
Toxicology Division

[REDACTED]
Technician, Laboratory of Genetic Toxicology
Toxicology Division

Report Preparation:

[REDACTED] D.V.M., Ph.D.
Senior Scientist, Laboratory of Genetic Toxicology
Toxicology Division

This document is not the property of EFSA and is provided for your full effect to the right of public access to documents under EU law.
The document may be subject to rights such as intellectual property and copy rights of third parties.
Furthermore, this document may fall under a regulatory data protection regime.
Consequently, any publication, distribution, reproduction and any commercial exploitation and use
of this document may therefore be prohibited and violate the rights of the owner.

QUALITY ASSURANCE AUTHORIZATION

HR-001: DNA Repair Test (Rec-Assay)

(IET 94-0141)

Report

	Inspection date	Report date to the study director	Report date to the administrator
Protocol	1/10/1995	1/10/1995	1/11/1995
	2/ 2/1995	2/ 2/1995	2/ 2/1995
Study procedure	2/14/1995	2/14/1995	2/14/1995
	2/15/1995	2/15/1995	2/15/1995
Raw data	2/20-23/1995	2/23/1995	2/23/1995
Report	2/20-23/1995	2/23/1995	2/23/1995
	3/14/1995	3/14/1995	3/14/1995

By the above inspections, it was assured that the reported methods and procedures were found to describe those used and the results to reflect the raw data generated during the conduct of this study accurately.

Quality Assurance Manager:


 D.V.M., Ph.D.,

Mar. 14, '95
 Date

D.J.C.V.P.
 Chief, Quality Assurance Unit

CONTENTS

	Page
TITLE PAGE -----	1
STATEMENT OF DATA CONFIDENTIALITY CLAIMS -----	2
GLP STATEMENT -----	3
FLAGGING STATEMENT -----	4
QUALITY ASSURANCE AUTHORIZATION -----	9
CONTENTS -----	10
1. SUMMARY -----	12
2. OBJECTIVE -----	13
3. TEST SUBSTANCE -----	13
4. MATERIALS AND METHODS	
1) Strains of bacteria -----	13
2) Examination of tester strains -----	14
3) Preparation of spore of tester strains -----	14
4) S9 fraction and co-factor solution -----	15
5) Preparation of the test substance solution and dose levels -----	16
6) Experimental procedures	
(1) Rec-assay without metabolic activation -----	17
(2) Rec-assay with metabolic activation -----	18
7) Criteria of a valid test -----	18

CONTENTS

	Page
8) Evaluation of results -----	19
5. RESULTS -----	20
6. CONCLUSION -----	21
7. REFERENCES -----	21
Table 1. Results of DNA repair test -----	22

This document is not the property of EFSA and is provided for giving full effect to the right of public access to documents under EU law.
The document may be subject to rights such as intellectual property and copyright protection.
Furthermore, this document may fall under a regulatory data protection regime.
Consequently, any publication, distribution, reproduction and/or publishing and any commercial exploitation and use
of this document or its contents without the permission of the owner
of this document may therefore be prohibited and violate the rights of its owner.

1. SUMMARY

DNA repair test (Rec-assay) with *Bacillus subtilis* strains of H17 and M45 was performed to evaluate the DNA-damaging activity of HR-001. HR-001 induced a growth inhibitory zone of 1 mm in diameter at the highest dose of 240 μ g/disk in the recombination-deficient strain M45 with liver metabolic activation system. On the other hand, HR-001 did not induce any growth inhibitory zone in either the recombination-proficient strain H17 with metabolic activation system or both the strains of M45 and H17 without metabolic activation system. The differences of growth inhibitory zones between the strains H17 and M45 were 1 mm or less which were the same degree as those observed in a negative control with kanamycin.

It was concluded that under the conditions used in this experiment, HR-001 did not have DNA-damaging activity in the bacteria.

2. OBJECTIVE

The purpose of this study was to evaluate the DNA-damaging activity of HR-001 in the bacteria.

3. TEST SUBSTANCE

Name: HR-001
Lot No.: 940908-1
Purity: 95.68%
Appearance at normal temperature: White crystal
Melting point: 200°C
Solubility: Water 12 g/l (25°C)
Storage condition: Dark cold room (5°C)

4. MATERIALS AND METHODS

1) Strains of bacteria

Recombination-wild (*rec⁺*) strain H17 and recombination-deficient (*recE⁻*) strain M45 of *Bacillus subtilis* were used to test DNA-damaging potential of the test substance. Both strains were obtained from Dr. [REDACTED] National Institute of Genetics, Mishima, Japan on March 26, 1973.

2) Examination of tester strains

The following characters of the tester strains were checked at the time of preparation of spore:

- (1) UV sensitivity (*recE*).
- (2) Response to negative and positive control chemicals.

3) Preparation of spore of tester strains

Each tester strain which has been stored at -80°C was inoculated with B2 medium (1% meat extract, 1% polypeptone, and 0.5% NaCl, pH 7) and incubated at 37°C with shaking. The overnight cultured cells were centrifuged and transferred into modified Schaeffer's medium (1.6% nutrient broth, 0.2% KCl, 0.1% glucose, 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 mM $\text{Ca}(\text{NO}_3)_2$, 1 μM FeSO_4 , and 0.1 mM MnCl_2). The cells were incubated at 37°C for 3 days for the strain H17 or 5 days for the strain M45. Sporulated cells were washed with 1/15 M phosphate buffer (pH 7.4) and then treated with lysozyme (100,000 unit/mg, MERCK Japan) at a final concentration of 2 mg/ml in Tris-buffer (10 mM Tris-HCl, 10 mM EDTA, pH 8.0) at 37°C for 30 min. Sodium dodecyl sulfate (SDS, Wako Pure Chemical Industries Ltd., Japan) was added at a final concentration of 1% and incubated at 37°C for 30 min. The obtained spores were washed several times with sterile pure water (Milli-RO-10, Milli-Q, Nippon Millipore Ltd.,

(IET 94-0141)

(Japan) and suspended in sterile pure water for storage at 4°C. Spores of strains M45 and H17 which were prepared on February 8, 1995 were used in this experiment.

4) S9 fraction and co-factor solution

S9 fraction with the following data was purchased from Kikkoman Corporation (Japan) on September 14, 1994 and stored at -80°C:

- (1) Animal: Sprague-Dawley rat (Slc:SD)
- (2) Sex: male
- (3) Age: 7 weeks old
- (4) Body weight: 188 - 238 g
- (5) Inducer: phenobarbital (PB: Wako Pure Chemical Industries Ltd., Japan)
5,6-benzoflavone (BF: Aldrich Chemical Co., Inc., U.S.A.)
- (6) Route of administration: Intraperitoneal injection
- (7) Dosage:
- | | |
|--------|-----------------------------|
| Day 1: | PB 30 mg/kg |
| Day 2: | PB 60 mg/kg |
| Day 3: | PB 60 mg/kg and BF 80 mg/kg |
| Day 4: | PB 60 mg/kg |
- (8) Protein content: 23.10 mg/ml
- (9) P-450 content: 1.26 nmol/mg protein

(10) Date of preparation: August 26, 1994

(11) Lot No.: RAA-314

(12) Sterility test: pass

(13) Enzyme activity measured by mutagenicity: good

The enzyme activity of this fraction was checked again by mutagenicity of 7,12-dimethylbenz(a)anthracene (Sigma Chemical Co., U.S.A., 95%) and 2-aminoanthracene (Wako Pure Chemical Industries Ltd., Japan, 96.5%) against *S. typhimurium* TA100 and TA98 in advance. The sterility of S9 fraction was also confirmed again in advance.

The components of co-factor solution were 8 mM MgCl₂, 33 mM KCl, 20 mg/ml of glucose-6-phosphate, 40 mg/ml of NADH, 40 mg/ml of NADPH, and 100 mM of sodium phosphate buffer (pH 7.4).

5) Preparation of the test substance solution and dose levels

The solubility in water of HR-001 was known to be 12 mg/ml, while it was insoluble in DMSO at this concentration. Therefore, sterile water was used as a solvent. A dose of 12 mg/ml, i.e., 240 μ g/disk, was employed as the highest dose, and 2-fold serial dilutions of the test substance were performed with sterile water for lower doses. The experiment was carried out at 6 dose levels (7.5, 15, 30, 60, 120, 240 μ g/disk). The solution of the test substance was prepared immediately before experiment.

6) Experimental procedures

(1) Rec-assay without metabolic activation

Molten B2 top agar medium (B2 medium supplemented with 0.8% agar, Wako Pure Chemical Industries Ltd., Japan, Lot No. PTE 7487) was prepared and kept at 45°C. In an empty 90-mm Petri dish, 0.1 ml of spore suspension (3×10^7 /ml) of tester strain were placed. Then, 5 ml of the above molten B2 top agar medium were poured in and mixed uniformly. Plates were left at room temperature until agar medium solidified.

A disk (8 mm in diameter and 1 mm in thickness) of paper filter was soaked with 20 μ l of the solution of the test substance and placed on the prepared spore agar plate. Kanamycin (Banyu Seiyaku Co., Japan, 1 g titer/vial) at 0.2 μ g/disk was used as a negative control chemical. Mitomycin C (Kyowa Hakko Kogyo Co., Ltd., Japan, 2 mg titer/vial) at 0.01 μ g/disk was used as a positive control chemical. In addition, a solvent control (20 μ l of sterile water/disk) was included in the experiment. Duplicate plates were used for each experimental point. Diameter of a growth inhibitory zone of each strain was measured after incubation at 37°C for 24 hr.

(2) Rec-assay with metabolic activation

Molten B2 top agar medium was prepared and kept at 45°C. In an empty 90-mm Petri dish, 0.1 ml of spore suspension (3×10^7 /ml) of tester strain and 0.05 ml of S9 fraction were placed. Then, 5 ml of molten B2 agar medium was poured in and mixed uniformly. Plates were left at room temperature until agar medium solidified.

A disk (8 mm in diameter and 1 mm in thickness) of paper filter was soaked with 20 μ l of the solution of the test substance and 20 μ l of co-factor solution and then placed on the prepared spore agar plate. 3-Amino-1,4-dimethyl-5H-pyrindo [4,3-b] indole (Trp-p-1, Wako Pure Chemical Industries Ltd., Japan) at 5 μ g/disk was used as a positive control. In addition, a solvent control (20 μ l of sterile water and 20 μ l of a co-factor solution/disk) was included in the experiment. Duplicate plates were made for each experimental point. Diameter of a growth inhibitory zone of each strain was measured after incubation at 37°C for 24 hr.

7) Criteria of a valid test

An assay is considered acceptable for evaluation of the test results only if all of the criteria listed below are satisfied;

(1) Growth inhibition is not observed in solvent controls of either strain.

(IET 94-0141)

(2) In a positive control, growth inhibitory zone in M45 is larger than that of H17, and the difference in diameter is 5 mm or more.

(3) In a negative control, the difference in diameter of growth inhibitory zone between the strains is 4 mm or less.

8) Evaluation of results

(1) Results are judged negative when the test substance causes no growth inhibition in either strain.

(2) In the case that the test substance causes growth inhibition at least in one strain, results are judged positive when growth inhibitory zone of M45 is larger than that of H17, and the difference in diameter was 5 mm or more at one or more dose levels that caused growth inhibitory zones of 4 mm or less in diameter in the H17 (rec⁺) strain. In this case, a re-test will be conducted to confirm reproducibility of the positive result.

5. RESULTS

As shown in Table 1, HR-001 induced a growth inhibitory zone of 1 mm in diameter at the highest dose of 240 μ g/disk in the recombination-deficient strain M45 with liver metabolic activation system. The differences of growth inhibitory zones between the strains H17 and M45 were 1 mm or less. On the other hand, HR-001 did not induce any growth inhibitory zone in either the recombination-proficient strain H17 with metabolic activation system or both the strains of M45 and H17 without metabolic activation system.

In the negative control plates treated with kanamycin, the difference of growth inhibitory zones between M45 and H17 strains was 2-3 mm. On the other hand, positive controls of mitomycin C without metabolic activation system and Trp-p-1 with metabolic activation system caused large inhibitory zones in the strain M45 when compared with those observed in H17. Actually, the differences of growth inhibitory zones induced by mitomycin C and Trp-p-1 were 19 mm and 11-12 mm in diameter, respectively. In a solvent control, no growth inhibitory zone was observed in either strain. These results indicated that this assay met all evaluation criteria.

6. CONCLUSION

HR-001 induced a growth inhibitory zone of 1 mm in diameter at the highest dose of 240 μ g/disk in the recombination-deficient strain M45 ^{without} with liver metabolic activation system. On the other hand, HR-001 did not induce any growth inhibitory zone in either the recombination-proficient strain H17 with metabolic activation system or both the strains of M45 and H17 without metabolic activation system. The differences of growth inhibitory zones between the strains H17 and M45 were 1 mm or less which were same degree as those observed in a negative control with kanamycin.

It was concluded that under the conditions used in this experiment, HR-001 did not have DNA-damaging activity in the bacteria.

7. REFERENCES

- 1) Kada, T., K. Hirano and Y. Shirasu: Chemical Mutagens, vol. 6, (Eds.) F. J. de Serres and A. Hollaender, 149-173, 1980.
- 2) Hirano, K., T., Hagiwara, Y. Ohta, H. Matsumoto, and T. Kada: Mutation Res., 97, 339-347, 1982.

Table I. Results of DNA repair test

Compound	Dose (μ g/ disk)	S9 fraction (-)			S9 fraction (+)			
		Inhibitory zone* (mm)		Differ- ence** (mm)	Inhibitory zone* (mm)		Differ- ence** (mm)	
		M45	H17		M45	H17		
Solvent control (H ₂ O)		0	0	0	0	0	0	
		0	0	0	0	0	0	
HR-001	7.5	0	0	0	0	0	0	
		0	0	0	0	0	0	
	15	0	0	0	0	0	0	
		0	0	0	0	0	0	
	30	0	0	0	0	0	0	
		0	0	0	0	0	0	
	60	0	0	0	0	0	0	
		0	0	0	0	0	0	
	120	0	0	0	0	0	0	
		0	0	0	0	0	0	
	240	1	0	1	0	0	0	
		0	0	0	0	0	0	
	Negative control (Kanamycin)	0.2	8	6	2			
			9	6	3			
Positive control (Mitomycin C)	0.01	20	1	19				
		20	1	19				
Positive control (Trp-P-1)	5				11	0	11	
					12	0	12	

* Diameter of growth inhibitory zone subtracted the diameter of disk (8 mm).

** Diameter of growth inhibitory zone in M45 strain subtracted that in H17 strain.