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Hoechst

Hoechst Aktiengesellschaft
Pharma Forschung Toxikologie

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CAS-Nr.: 81-33-4

Perylimid

Study of the mutagenic potential in strains of Salmonella typhimurium (Ames Test) and Escherichia coli

Confidential Business Information (CBI) Claims Withdrawn by Clariant on February 27, 2019

Corresponds to Study #14 in Attachment A of Transmittal Memo on CBI
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CONTENTS

1. SUMMARY

Perylimid was tested for mutagenicity with the strains TA 100, TA 1535, TA 1537, TA 1538, TA 98 of Salmonella typhimurium and Escherichia coli WP2uvrA.

The mutagenicity studies were conducted in the absence and in the presence of a metabolizing system derived from rat liver homogenate. A dose range of 6 different doses from 4 µg/plate to 5 000 µg/plate was used.

Control plates without mutagen showed that the number of spontaneous revertant colonies was similar to that described in the literature. All the positiv control compounds gave the expected increase in the number of revertant colonies.

Toxicity: The test compound proved to be not toxic to the bacterial strains. 5 000 µg/plate was chosen as top dose level for the mutagenicity study.

Mutagenicity: In the absence of the metabolic activation system the test compound did not show a dose dependent increase in the number of revertants in any of the bacterial strains. Also in the presence of metabolic activation system, treatment of the cells with **Perylimid** did not result in relevant increases in the number of revertant colonies.

Summarizing, it can be stated that **Perylimid** is not mutagenic in these bacterial test systems neither with nor without exogenous metabolic activation at the dose levels investigated.

2. INTRODUCTION

This report describes experiments performed in a short term test using the procedure of the Salmonella / mammalian-microsome-mutagenicity test (Ames Test) (1,2) to assess the mutagenic potential of the test-material in amino acid-dependent strains of Salmonella typhimurium and a strain of Escherichia coli described by Green (3). By the use of liver homogenate the test takes into account the mammalian metabolism of the compound to be tested. The requirement for metabolic activation was investigated by incorporating into the test an activation system by nicotinamide-adenine dinucleotide phosphate ($NADP^+$)-cytochrome P_{450} dependent mixed function oxidase enzymes of the liver. The 9000 g supernatant of rat liver homogenate has been shown to be very useful in metabolic activation of foreign compounds. The animals were pretreated with Aroclor 1254 as an inducer of several drug metabolizing enzymes (4).

In the Ames test with Salmonella typhimurium strains the effect of the test compound upon the number of back mutations to histidine prototrophy using histidine auxotrophic mutants is investigated. Using Escherichia coli WP2uvrA, a tryptophan dependent auxotroph strain, mutagenicity is based on reversion to tryptophan independence. The strains TA 100 and TA 1535 were originally derived by a substitution mutation, the strains TA 1537, TA 1538 and TA 98 by frame shift mutations from histidine prototrophic bacteria. All five Salmonella strains are deficient in the complete structure of their lipopolysaccharide layer and in DNA excision repair system (2). TA 98 and TA 100 possess a modified postreplication DNA repair system which frequently causes an increase in the rate of mutations (5). Strain WP2uvrA carries a defect in one of the genes for tryptophan biosynthesis and is deficient in the uvrA system of DNA repair. The reversion can be induced by a base change (substitution).

3. GENERAL

Study-No.	:	83.0528
Test compound	:	**Perylimid**
Ordered by	:	Hoechst, Farbenforschung P Gr. 2
Test system	:	Point mutation assay with bacteria
Test organisms		
Salmonella typhimurium	:	TA 100, TA 1535, TA 1537, TA 1538, TA 98
Escherichia coli	:	WP2uvrA
Initiation of the study	:	December 6, 1983
Termination of the study	:	December 16, 1983

R e s p o n s i b i l i t y

Industrial Toxicology	:	Dr. WEIGAND
Study director	:	Dr. JUNG
Quality assurance unit	:	Ap. Harston
Testing facilities and archives	:	Department of Toxicology Industrial Toxicology HOECHST AKTIENGESELLSCHAFT Post box 80 03 20 6230 Frankfurt 80

4. MATERIAL AND METHODS

4.1. Test compound

Name	:	**Perylimid**
Other names	:	Pelylen-3,4,9,10-tetracarbonsäurediimid
CAS - No.	:	81-33-4
Utilisation	:	pigment
Chemical nomenclature	:	anthra[2,1,9-def:6,5,10-d',e',f']diisoquinoline-1,3,8,10-2H,9H-tetrone
Molecular formular	:	$C_{24}H_{10}N_2O_4$
Purity	:	99 %
Appearence	:	black powder
Melting point	:	350° C
Molecular weight	:	390
pH - Value	:	7
Batch No.	:	5942/2
Date of submission	:	October 10, 1983
Storage conditions	:	dark at 20° C

At the day of the experiment the test substance was suspended in DMSO at appropriate concentrations.

4.2 Preparation and storage of a liver homogenate fraction ("S-9")

Male Sprague Dawley rats (200 - 300 g) receive a single intraperitoneal injection of Aroclor 1254 (500 mg/kg bodyweight) 5 days before sacrifice. Preparation is performed at 0 to 4°C using cold sterile solution and glassware. The livers from at least 5 - 6 animals are removed and pooled, washed in 150 mM KCl (approximately 1 ml/g wet livers). The washed livers are cut into small pieces and homogenized in three volumes of KCl. The homogenate is centrifuged at 9000 g for 10 minutes. The supernatant is the S-9 fraction. It is devided into small portions, rapidly frozen and stored at -80°C for not longer than three months.

4.3 Preparation of S-9 Mix

Sufficient S-9 fraction is thawed immediately before each test at room temperature. One volume of S-9 fraction is mixed with 9 volumes of the S-9 cofactor solution and kept on ice until used. This preparation is termed S-9 Mix. The concentrations of the different compounds in the S-9 Mix are:

8 mM $MgCl_2$
33 mM KCl
5 mM glucose-6-phosphate
4 mM NADP+
100 mM phosphate buffer pH 7,4

4.4 Bacteria

Bacteria are grown overnight in nutrient broth (25 g Oxoid Nutrient Broth No 2 /liter) at 37°C. The suitable amount of bacteria in the cell suspension is checked by nephelometry. For inoculation, stock cultures which are stored at -80°C, are used. The compound is tested with the strains Salmonella typhimurium TA 98, TA 100, TA 1535, TA 1537 and TA 1538 and E. coli WP2uvrA. Identification of the different bacterial strains is performed periodically as described (2,3).

4.5 Toxicity experiments and dose range finding

Preliminary toxicity tests were performed with all tester strains using a small number of plates to calculate an appropriate dose range. A reduced rate of spontaneously occuring colonies as well as visible thinning of the bacterial lawn were used as indicator for toxicity. Thinning of the bacterial lawn was controlled microscopically.

In combination with the main experiment, toxicity testing was performed as follows: 0,1 ml of the different dilutions of the test compound were thoroughly mixed with 0,1 ml of 10^{-6} dilution of the overnight culture of TA 100 and plated with histidine and biotin rich top agar (3 plates per dose). The solvent control is compared with the number of colonies per plate in the presence of the test compound. Results are given as a ratio of these values (= surviving fraction).

4.6 Mutagenicity test

Top agar is prepared for the Salmonella strains by mixing 100 ml agar (0,6 % agar, 0,5 % NaCl) with 10 ml of a 0,5 mM histidine-biotin solution. With E. coli histidine is replaced by tryptophan (2,5 ml, 0,5 mM). The following ingredients are added (in order) to 2 ml of molten top ager at 45°C:

0.1 ml of an overnight nutrient broth culture of the bacterial tester strain
0.1 ml test compound solution
0.5 ml S-9 Mix (if required) or buffer

After mixing, the liquid is poured into a petridish with minimal agar (1,5 % agar, Vogel-Bonner E medium with 2 % glucose). After incubation for 48 to 72 hours at 37°C in the dark, colonies (his^+ revertants) are counted.

4.7 Positive controls

Positive control plates were included for each strain. The following substances were used as positive controls.

a) without metabolic activation:

Na-azide: TA 100, TA 1535;
9-Aminoacridine: TA 1537
2-Nitrofluorene: TA 98, TA 1538
N-Methyl-N'-nitro-N-nitrosoguanidine (MNNG): WP2uvrA

b) with metabolic activation:

Benzo[a]pyrene: TA 98, TA 100, TA 1535, TA 1537, TA 1538, WP2uvrA
2-Aminoanthracene: TA 98, TA 100, TA 1535, TA 1537, TA 1538, WP2uvrA

5. RESULTS

Perylimid was tested for mutagenicity with Salmonella typhimurium strains TA 100, TA 1535, TA 1537, TA 1538, TA 98 and E. coli WP2uvrA in the absence and presence of a metabolic activation system. The results obtained with the test material and positive control compounds are presented in table 1 to 8a. The number of colonies per plate with each strain as well as mean values of 3 plates, corrected to the next whole number are given.

5.1 Sterility checks and control plates

Sterility of S-9 Mix and the test compound was indicated by the absence of contamination on the test material and S-9 Mix sterility check plates. Control plates (background control and positive controls) gave the expected number of colonies.

5.2 Toxicity test

The test compound was tested at doses of 4 to 10 000 µg/plate (table 1) and proved to be not toxic to the bacteria. For reason of heavy precipitation of the test compound the bacterial lawn could only be evaluated up to a dose level of 2 500 µg/plate. Visible precipitation of the test compound on the plates has been observed at 500 µg/plate.

For mutagenicity testing 5 000 µg/plate was chosen as the highest dose.

5.3 Mutagenicity test with Perylimid

The test compound did not cause a significant increase in the number of revertant colonies with any of the tester strains neither in the absence nor presence of S-9 Mix. No dose dependent effect was obtained (table 2 - 7).

It is concluded that the test substance is not mutagenic in these bacterial test systems neither in the absence nor in the presence of an exogenous metabolizing system.

This test was performed according to the methods described. No unforeseen circumstances were observed which have affected the quality and integrity of this report.

Dr. Jg/Lu

Quality assurance unit

Department of Toxicology
-Industrial Toxicology-
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6. TABLES

Table 1: Toxicity experiment and dose range finding with **Perylimid**

Number of revertant colonies obtained with
Salmonella typhimurium strains TA 100, TA 1535, TA 1537, TA 1538, TA 98
and Escherichia coli WP2uvrA

Dose[1] µg/plate	Metabolic activation	Strain of Salmonella typhimurium					E. coli
		TA 100	TA 1535	TA 1537	TA 1538	TA 98	WP2uvrA
0	-	159	11	11	8	15	35
4	-	139	17	6	17	16	26
20	-	69	17	6	12	15	26
100	-	125	18	8	6	15	30
500 p	-	136	13	6	6	21	38
2 500 p	-	118	15	1	9	21	26
10 000 p	-	141	17	2	8	17	42
0	+	149	12	11	9	27	32
4	+	149	12	5	18	36	24
20	+	148	13	7	13	28	36
100	+	174	7	11	19	24	32
500 p	+	189	10	12	14	21	37
2 500 p	+	176	13	5	21	17	40
10 000 p	+	159	6	14	7	17	32

[1] : suspended in 100 µl DMSO

\- : absence
\+ : presence
p : visible precipitation of the test compound on the plates

Table 2: Mutagenicity experiment with Perylimid with and without metabolic activation

TA 100

Number of revertant colonies per plate and mean values using Salmonella typhimurium strain TA 100

Dose[1] µg/plate	Metabolic activation	Mean value	Colonies per plate	Surviving fraction
0	-	152	167, 138, 150	1.0
4	-	161	169, 174, 141	1.0
20	-	140	141, 137, 143	1.1
100	-	151	138, 162, 152	1.0
500 p	-	137	140, 152, 118	1.1
2 500 p	-	139	160, 137, 119	0.9
5 000 p	-	140	160, 136, 124	1.2
0	+	145	161, 152, 123	1.0
4	+	151	172, 156, 124	1.1
20	+	137	159, 129, 123	0.8
100	+	155	171, 131, 162	1.0
500 p	+	161	184, 136, 162	1.3
2 500 p	+	129	147, 111, 129	1.1
5 000 p	+	145	173, 136, 127	1.1

[1] : suspended in 100 µl DMSO

\- : absence
\+ : presence
p : visible precipitation of the test compound on the plates

Table 3: Mutagenicity experiment with Perylimid with and without metabolic activation

TA 1535

Number of revertant colonies per plate and mean values using Salmonella typhimurium strain TA 1535

Dose[1] µg/plate	Metabolic activation	Mean value	Colonies per plate
0	-	17	17, 15, 18
4	-	16	15, 20, 14
20	-	18	15, 18, 22
100	-	23	25, 21, 24
500 p	-	16	16, 12, 19
2 500 p	-	17	16, 20, 15
5 000 p	-	21	20, 30, 14
0	+	10	8, 7, 15
4	+	10	10, 10, 11
20	+	9	7, 10, 9
100	+	10	10, 12, 7
500 p	+	10	7, 10, 14
2 500 p	+	9	11, 8, 9
5 000 p	+	11	10, 17, 6

1 : suspended in 100 µl DMSO

\- : absence
\+ : presence
p : visible precipitation of the test compound on the plates

Table 4: Mutagenicity experiment with **Perylimid** with and without metabolic activation

TA 1537

Number of revertant colonies per plate and mean values using Salmonella typhimurium strain TA 1537

Dose[1] µg/plate	Metabolic activation	Mean value	Colonies per plate
0	-	7	4, 8, 10
4	-	8	14, 3, 6
20	-	8	7, 4, 12
100	-	5	6, 3, 6
500 p	-	6	6, 7, 4
2 500 p	-	5	6, 5, 7
5 000 p	-	7	3, 6, 11
0	+	7	8, 8, 6
4	+	8	10, 8, 6
20	+	9	8, 12, 8
100	+	7	8, 8, 5
500 p	+	8	10, 6, 9
2 500 p	+	7	6, 12, 3
5 000 p	+	8	6, 12, 7

[1] : suspended in 100 µl DMSO

\- : absence
\+ : presence
p : visible precipitation of the test compound on the plates

Table 5: Mutagenicity experiment with Perylimid with and without metabolic activation

TA 1538

Number of revertant colonies per plate and mean values using Salmonella typhimurium strain TA 1538

Dose[1] µg/plate	Metabolic activation	Mean value	Colonies per plate
0	-	12	14, 14, 7
4	-	9	10, 9, 8
20	-	11	15, 11, 8
100	-	7	5, 8, 9
500 p	-	7	4, 7, 11
2 500 p	-	7	5, 10, 7
5 000 p	-	9	12, 8, 6
0	+	18	20, 14, 20
4	+	16	16, 18, 15
20	+	12	9, 16, 10
100	+	16	11, 25, 13
500 p	+	13	13, 13, 13
2 500 p	+	12	7, 15, 14
5 000 p	+	9	11, 9, 6

1 : suspended in 100 µl DMSO

\- : absence
\+ : presence
p : visible precipitation of the test compound on the plates

Table 6: Mutagenicity experiment with Perylimid with and without metabolic activation

TA 98

Number of revertant colonies per plate and mean values using Salmonella typhimurium strain TA 98

Dose[1] µg/plate	Metabolic activation	Mean value	Colonies per plate
0	-	14	9, 16, 16
4	-	16	12, 19, 17
20	-	19	16, 24, 18
100	-	16	16, 13, 19
500 p	-	19	18, 18, 20
2 500 p	-	14	18, 10, 15
5 000 p	-	16	11, 15, 21
0	+	23	26, 21, 21
4	+	26	30, 28, 21
20	+	24	23, 24, 25
100	+	21	23, 22, 19
500 p	+	23	19, 21, 28
2 500 p	+	25	29, 22, 25
5 000 p	+	14	17, 14, 11

[1] : suspended in 100 µl DMSO

\- : absence
\+ : presence
p : visible precipitation of the test compound on the plates

Table 7: Mutagenicity experiment with Perylimid with and without metabolic activation

WP2uvrA

Number of revertant colonies per plate and mean values using Escherichia coli strain WP2uvrA

Dose[1] µg/plate	Metabolic activation	Mean value	Colonies per plate
0	-	37	42, 39, 29
4	-	32	34, 37, 25
20	-	40	38, 47, 34
100	-	37	48, 29, 33
500 p	-	34	34, 38, 29
2 500 p	-	37	31, 47, 33
5 000 p	-	31	36, 34, 24
0	+	44	38, 47, 46
4	+	40	57, 34, 28
20	+	36	42, 37, 28
100	+	39	47, 35, 36
500 p	+	45	46, 44, 25
2 500 p	+	33	33, 28, 39
5 000 p	+	36	40, 29, 39

[1] : suspended in 100 µl DMSO

\- : absence
\+ : presence
p : visible precipitation of the test compound on the plates

Table 8: mutability (positive controls) and sterility test of the experiment with **Perylimid**

Number of revertant colonies per plate and mean values using Salmonella typhimurium strains and Escherichia coli

Strain	Compound	Dose µg/plate	Metab. activ.	Mean value	Colonies per plate
TA 100	Sodium azide	1	-	529	514, 549, 524
TA 1535	Sodium azide	1	-	406	395, 395, 427
TA 1537	9-Aminoacridine	50	-	344	439, 273, 319
TA 1538	2-Nitrofluorene	5	-	747	850, 615, 775
TA 98	2-Nitrofluorene	5	-	487	530, 445, 485
WP2uvrA	MNNG	2.5	-	590	635, 533, 602
-	**Perylimid**	5 000	-	0	0, 0, 0

- : absence

Table 8a: mutability (positive controls) and sterility test of the experiment with **Perylimid**

Number of revertant colonies per plate and mean values
using Salmonella typhimurium strains and Escherichia coli

Strain	Compound	Dose µg/plate	Metab. activ.	Mean value	Colonies per plate
TA 100	2-Aminoanthracene	0.5	+	579	579, 558, 599
TA 1535	2-Aminoanthracene	1	+	146	148, 134, 156
TA 1537	2-Aminoanthracene	1	+	103	98, 97, 113
TA 1538	2-Aminoanthracene	0.5	+	311	417, 305, 210
TA 98	2-Aminoanthracene	0.5	+	320	229, 379, 351
WP2uvrA	2-Aminoanthracene	10	+	404	400, 424, 388
TA 100	Benzo[a]pyrene	10	+	559	480, 712, 485
TA 1535	Benzo[a]pyrene	10	+	22	27, 16, 22
TA 1537	Benzo[a]pyrene	10	+	184	174, 188, 189
TA 1538	Benzo[a]pyrene	10	+	257	229, 273, 268
TA 98	Benzo[a]pyrene	10	+	479	487, 525, 425
WP2uvrA	Benzo[a]pyrene	10	+	75	62, 81, 83
-	S-9 mix	500 µl	+	0	0, 0, 0
-	**Perylimid**	5 000 µg	+	0	0, 0, 0

+ : presence

5. REFERENCES

1) B.N. Ames, W.W. Durston, E. Yamasaki and F.D. Lee, Carcinogens are mutagens. A simple test system combining liver homogenate for activation and bacteria for detection, Proc. Nat. Acad. Sci. USA 70 (1973) 2281 - 2285.

2) B.N. Ames, J. McCann and E. Yamasaki: Methods for detecting carcinogens and mutagens with the Salmonella / mammalian-microsome mutagenicity test, Mutation Res. 31 (1975) 347 - 364.

3) M.H.L. Green and W.J. Muriel: Mutagen testing using trp^+ reversion in Escherichia coli, Mutation Res. 38 (1976) 3 - 32.

4) A.P. Alvares, D.R. Bickers and A. Kappas: Polychlorinated biphenyls: a new type of inducer of cytochrome P 448 in the liver. Proc. Nat. Acad. Sci. USA 70 (1973) 1321 - 1325.

5) J. McCann, N.E. Springarn, J. Kobory and B.N. Ames: Detection of carcinogens as mutagens: bacterial tester strains with R factor plasmids, Proc. Nat. Acad. Sci. USA 72 (1975) 979 - 983.

Hoechst

Hoechst Aktiengesellschaft
Pharma Research
Quality Assurance (GLP)
December 23, 1983

Title : Perylimid
Study of the mutagenic potential in strains of Salmonella typhimurium (Ames Test) and Escherichia coli
Date : December 20, 1983

Study No. : 83.0528

This study was periodically inspected and properly signed records of these inspections were submitted to testing facility management and the study director as shown below:

Inspection	Report
05.12.1983	05.12.1983
14.12.1983	14.12.1983
23.12.1983	23.12.1983

Pharma Research
Quality Assurance (GLP)

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