"UKRAIN 5 mg Ampullen": Salmonella Typhimurium Reverse Mutation Test

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"UKRAIN 5 mg Ampullen": Salmonella Typhimurium Reverse Mutation Test

Report for
Nowicky Pharma
A-1040 Wien, Margaretenstraße 7

Department of Toxicology

Division of Life Sciences

Confidential

"UKRAIN 5 mg Ampullen": Salmonella Typhimurium Reverse Mutation Test

CONTENTS

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"UKRAIN 5 mg Ampullen": Salmonella Typhimurium Reverse Mutation Test

SUMMARY

Method

"UKRAIN 5 mg Ampullen" was tested for mutagenic activity with the *"Salmonella typhimurium Reverse Mutation Test"* (Ames Test). The study was conducted in accordance with the OECD-guideline 471 and EEC Guideline 92/69, part B.14.

The test substance, diluted in water, was tested at concentrations ranging from 1.2 µl to 100 µl per plate according to the *"direct plate incorporation method"* without external metabolisation as well as with an external metabolising system (S9-mix). As test system the bacterial strains Salmonella typhimurium TA97a, TA98, TA100, TA102 and TA1535 were used. Negative and positive controls were included. An independent repetition of the experiment was performed.

Results

Positive controls:
All positive control groups showed significantly increased mutation frequencies which demonstrate the sensitivity of the test system.

Test substance:
Solubility:
The test substance was freely miscible with water.

Toxicity:
The test substance was not toxic to the Salmonella strains used. No reduced or missing bacterial background lawn was observed.

Mutagenicity:
In none of the concentrations tested and with none of the strains used a statistically significant increase of the mutation frequency to more than twice the amount of the control samples was obtained. Metabolic activation did not change these results.

Conclusion

According to the results obtained in this study, **"UKRAIN 5 mg Ampullen" is non-mutagenic in the Ames test** with the strains TA97a, TA98, TA100, TA102 and TA1535 up to 100 µl per plate.

15 Jan. 1999

Mag. Peter Weniger
Study Director

PP 01 18

Dr. Norbert Bornatowicz
Head of Toxicology Department

GENERAL INFORMATION

Responsible personnel:

Study director, test and reference substance preparation, report:	Mag. Peter Weniger.
Performance of the test:	Dipl. Ing. Wolfgang Fiedler, Mag. Peter Weniger.
Quality assurance:	Dr. Heinz Hofer.

Sponsor:

Nowicky Pharma, A-1040 Wien, Margaretenstraße 7.

Study monitor: Dr. J. W. Nowicky.

Guidelines applied:

- OECD-Guideline 471. Genetic Toxicology: Salmonella typhimurium Reverse Mutation Test, 21 July 1997.
- EEC Guideline 92/69 part B.14: Mutagenicity, Salmonella typhimurium Reverse Mutation Assay.
- OECD Principles of Good Laboratory Practice, OECD Environment Health and Safety Publications, Series on Principles of Good Laboratory Practice and Compliance Monitoring No. 1, Paris 1998.

Internal study code: NO6

Time schedule:

Date of order:	18 September 1998
Date of protocol:	28 September 1998
Experimental starting date (preparing the overnight culture for the first experiment):	16 November 1998
Experimental completion date (last colony counting):	26 November 1998

Location of laboratories and archives:

Österreichisches Forschungszentrum Seibersdorf Ges.m.b.H., A-2444 Seibersdorf, Austria.

Archives:

All raw data, a copy of the final report and the test substance are retained for 10 years. The data and the test substance are not destroyed without the information of the sponsor.

Number of pages of this report: I-II, 1 - 27.

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

Statement concerning the study:

"UKRAIN 5 mg Ampullen": Salmonella Typhimurium Reverse Mutation Test

This study meets the requirements of the OECD Principles of Good Laboratory Practice, OECD Environment Health and Safety Publications, Series on Principles of Good Laboratory Practice and Compliance Monitoring No. 1, Paris 1998, with the exception that the solutions of the test substance were not analysed for stability and concentration of the test substance.

Seibersdorf, 15 January 1999

Mag. Peter Weniger
Study Director

QUALITY ASSURANCE UNIT

Statement concerning the study:

"UKRAIN 5 mg Ampullen":
Salmonella Typhimurium Reverse Mutation Test

The following inspections were made concerning this study:

date of inspection	inspected phases	date of report to the management
28 September 1998	protocol	28 September 1998
24 November 1998	experimental phase	24 November 1998
14 January 1999	report	14 January 1999

This report has been reviewed by the Quality Assurance Unit. The reported methods and procedures were found to describe those used and the results to constitute an accurate representation of the data recorded.

Seibersdorf, 15 January 1999

Dr. Heinz Hofer
Quality Assurance Unit

"UKRAIN 5 mg Ampullen": Salmonella Typhimurium Reverse Mutation Test

1. INTRODUCTION

This study was performed to determine a possible mutagenic action of "UKRAIN 5 mg Ampullen" with the Salmonella typhimurium reverse mutation test (Ames test). This test is sensitive to frameshift mutations as well as to base pair mutations. The performance of the test with and without an external metabolising system ensures the detection of the mutagenic action of the test substance itself as well as of its metabolites.

2. MATERIALS AND EXPERIMENTAL CONDITIONS

The compositions of the different solutions are shown in Appendix 1.

2.1. Test substance

2.1.1. Description by the sponsor

Name:	"UKRAIN 5 mg Ampullen"
Active ingredient:	Chelidonium majus L.-alkaloid-thiophosphoric acid derivative
CAS No.:	138069-52-0
Batch No.:	544324
Purity:	95.9 %
Certificate of analysis:	See Appendix 2
Concentration of the active ingredient:	1 mg/ml
Solubility in water:	miscible
Density:	0.9986
pH:	3.49
Storage:	ambient temperature, protected from light
Stability at storage conditions:	5 years
Expiry date:	June 2001

2.1.2. Description and characterisation by the test facility

Date of receipt:	28 September 1998
Label on the shipping container:	UKRAIN® Wirkstoff: Chelidonium majus L. Alkaloid - Thiophosphorsäurederivat 1 Ampulle a 5 ml 1 ampoule a 5 ml i.v. oder i.m. Injektion i.v. or i.m. injection 5 mg/5 ml Hersteller: Nowicky Pharma Margaretenstrasse 7 A-1040 Vienna
Appearance:	light brown solution
Storage:	room temperature, protected from light

2.2. Reference substances

- 2-Aminoanthracene, (Sigma, article A-1381. Practical grade).
- 7,12-Dimethylbenz[a]anthracene (Sigma, article D 3254)
- 1,8-Dihydroxy-anthraquinone (Danthron, Sigma, article D-5636, 95%).
- 2-Nitrofluorene (Aldrich, article N 1,675-4)
- Sodium azide (Sigma, article S-2002)
- 4-Nitro-o-phenylenediamine (Sigma, article N-9504).
- t-Butyl-hydroperoxide (Schuchard, article 814006, 70 % aqu. solution).

1,8-Dihydroxy-anthraquinone, 7,12-dimethylbenz[a]anthracene and 2-aminoanthracene are mutagenic only when activated by a metabolising system; 4-nitro-o-phenylenediamine, 2-nitrofluorene, t-butyl-hydroperoxide and sodium azide are directly acting mutagens.

Sodium azide and t-butyl-hydroperoxide were dissolved in sterile water. The other reference substances were prepared from stock solutions in dimethylsulfoxide.

2.3. Test and reference substance solutions - preparation

The test substance was used undiluted for the highest concentration group. The solutions for the lower concentrations were prepared by subsequent dilution of one volume of the higher concentrated solution with two volumes of water.

The test substance ampoules were opened and the test substance was diluted immediately before use. The time between preparing the test substance solutions and the last application to the bacteria was less than 3 hours.

4-Nitro-o-phenylenediamine, 1,8-dihydroxy-anthraquinone and 2- aminoanthracene were prepared from stock solutions in DMSO (dimethylsulfoxide).
Sodium azide and t-butyl-hydroperoxide were dissolved in sterile water.

2.4. Test system

Bacterial strains of Salmonella typhimurium TA97a, TA98, TA100, TA102 and TA1535, obtained from Prof. Bruce N. Ames, Berkeley, California, were used.
The bacteria were stored in small portions in a solution of 6 % DMSO in phosphate buffered saline in liquid nitrogen.
The actual batch of the strains was tested for ampicillin resistance (TA102: ampicillin/tetracycline resistance), UV-sensitivity and sensitivity against crystal violet, for spontaneous mutation frequencies and for sensitivities against the positive control substances in November 1997. The bacteria were stored frozen since that time.

The main genetic properties of these strains are:

Strain:	his-Mutation:	rfa:	uvrB:	pkM101:
TA97a	D6610	yes	yes	yes
TA98	D3052	yes	yes	yes
TA100	G46	yes	yes	yes
TA102	G428	yes	no	yes
TA1535	G46	yes	yes	no

D6610 and D3052 are frameshift mutations, G46 is a base pair mutation, G428 is an ochre-mutation.

The rfa mutation leads to a reduced lipopolysaccharide barrier in the cell wall and allows larger molecules to pass the cell wall. Bacteria with this mutation are sensitive to crystal violet.

UvrB results in a loss of the DNA-excision repair system. This increases the sensitivity to mutagenic influences. Bacteria with this mutation are sensitive to UV light.

The plasmide pkM101 also disturbs the ability of the bacteria to repair genetic damage and therefore increases their sensitivity to mutagens. Bacteria with this plasmide are resistant against ampicillin. TA102 is also resistant against tetracycline.

2.4.1. Justification for the bacterial strains used

S. typhimurium TA97a (or TA97 or TA1537), TA98, TA100, TA102 (or E. coli WP2) and TA1535 are required by the OECD guideline.

2.4.2. Conditions of cultivation

One day before the Ames test was performed, a small amount from each of the frozen bacterial cultures was transferred to nutrient broth. The liquid cultures were incubated overnight at 37 °C and then used for the exposure.

2.5. Metabolic system

The microsomal fraction of homogenised livers of rats treated once with 500 mg/kg of Aroclor 1254 was used. The preparation was from May 1998. Four days after treatment, the feed was withdrawn for one night. The livers of the animals were removed and homogenised in cold 0.15 mol/l KCl. Three ml of homogenate were obtained per gram of liver. Then the homogenate was centrifuged for 10 minutes at 9000 x g. The supernatant contained the microsomes. Small portions of the microsomes were stored in liquid nitrogen. Immediately before use they were thawed and mixed with the cofactor solution. The metabolic activity of the microsomes was verified by the positive control substances of each study and by the positive control substances listed in Table 7.

2.6. Exposure

2.6.1. Groups, concentrations, number of samples

The test substance was tested without as well as with an external metabolising system (S9-mix). The results were verified by a second, independent experiment.

samples	concentrations used	replicates
test substance	100, 33, 11, 3.7 and 1.2 µl/plate	3 samples
control (water)	100 µl	6 samples
positive control *	* (see below)	3 samples

Strain:	without S9	with S9
TA97a	4NOPD, 10 µg	DMBA, 5 µg
TA98	2NF, 2 µg	2AA, 1 µg
TA100	Sodium-azide, 2 µg	2AA, 2 µg
TA102	tBHPO, 50 µg	DHA, 50 µg
TA1535	Sodium-azide, 1 µg	2AA, 2 µg

4NOPD: 4-Nitro-o-phenylene-diamine
tBHPO: t-Butyl-hydroperoxide
2AA: 2- Aminoanthracene
DHA: 1,8-Dihydroxy-anthraquinone
DMBA: 7,12-Dimethylbenz[a]anthracene
2NF: 2-Nitrofluorene

2.6.2. Justification for the concentrations

The guidelines recommend 5 mg of 'pure' substances per plate for the highest concentration. As the test substance was not a 'pure' chemical but rather an aqueous formulation, the highest technical feasible amount, 100 µl, was taken for the high concentration group. The other concentrations were obtained by subsequent dilution to one third each.

2.6.3. Exposure technique

The exposure was performed according to the 'Plate Incorporation Assay', in which bacteria, test substance (and microsomes) are in contact on the plate without preceding incubation in the liquid state. The number of viable cells in the overnight-culture is in the range of 2×10^8 cells per ml.

For each sample the following solutions were combined:

- 0.1 ml of the overnight culture of the bacteria,

- 0.5 ml of S9-mix (or phosphate buffered saline for samples without metabolic activation),
- 0.1 ml of the appropriate test- or reference substance solution and
- 2 ml of top agar.

The combined solutions were mixed and spread over a plate with minimal agar (9 cm diameter). After the top agar had solidified, the plates were incubated at 37 °C until the colonies were visible (2 days).

2.7. Counting of colonies

The plates were counted visually by marking the colonies with a felt tipped pen. From plates with more than about 300 colonies a fraction of the total area was counted visually and the total amount of colonies was calculated. When more than one person was counting, each person counted equal numbers of plates of the control group and of each of the dosed groups.

2.8. Statistical methods

Means and standard deviation were calculated for the number of mutants in every concentration group.

For comparison of the control group and the test substance groups the analysis of variance was used followed by the test of Scheffé. For comparison of the control group and the positive control group, if the results were not clear beyond any doubt, the t-test was used. $p = 0.05$ was used as the significance level.

The criteria for a positive result are:
A reproducible statistically significant increase of the number of revertants to more than twice the amount of the spontaneous revertants for at least one of the concentrations.

2.9. Unforeseen events

No unforeseen events occurred which could impede the outcome of this study.

3. RESULTS

3.1. Properties of the bacteria

The properties of the strains are listed in Table 7. The used strains of Salmonella typhimurium showed the expected genetic properties and were sensitive against several mutagenic chemicals.

3.2. Reference substances

The positive control substances increased the mutation frequency statistically significantly (Table 1 to Table 5). As 2-aminoanthracene, 1,8-dihydroxy-anthraquinone and 7,12-Dimethylbenz[a]anthracene require metabolic activation for mutagenicity, the results of these substances demonstrate also the efficiency of the metabolising system.

The mutation frequencies of the negative control groups were in the usual range for the different strains.

3.3. Test substance

The means and standard deviations of the results are shown in Table 1 to Table 5, the counts of the individual plates in Table 6.

3.3.1. Solubility

The test substance was freely miscible with water.

3.3.2. Toxicity

The test substance was not toxic to the Salmonella strains used. No reduced or missing bacterial background lawn was observed.

3.3.3. Mutagenicity

This is the parameter of major interest, because a substance is called mutagenic in the Ames test when a reproducible statistically significant increase in the number of revertants to more than twice the amount of the spontaneous revertants for at least one of the concentrations occurs.

There was no statistically significant increase in the number of mutants to more than twice the amount of the negative controls in any of the tested bacterial strains at any of the tested concentrations. The addition of an external metabolising system did not change these results.

According to these results, **"UKRAIN 5 mg Ampullen" is not mutagenic in the Ames test** with the strains of Salmonella typhimurium TA97a, TA98, TA100, TA102 and TA1535 up to 100 µl per plate.

Responsible scientists:

15 Jan. 1999

Mag. Peter Weniger
Study director

P90118

Dr. Norbert Bornatowicz
Head of Toxicology Department

"UKRAIN 5 mg Ampullen": Salmonella Typhimurium Reverse Mutation Test

Table 1: Mean number of revertants per plate for strain TA97a

Mean, standard deviations SD and number of plates N.

Without metabolisation

first experiment				second experiment			
conc.	revertants / plate			conc.	revertants / plate		
µl/plate	mean	SD	N	µl/plate	mean	SD	N
100	148.3	5.0	3	100	144.0	2.6	3
33	142.3	5.5	3	33	133.0	7.0	3
11	152.7	5.5	3	11	133.0	18.2	3
3.7	154.3	15.0	3	3.7	151.0	14.8	3
1.2	166.3	10.7	3	1.2	125.3	7.0	3
solvent	162.7	14.6	6	solvent	135.7	16.6	6
positive	1266.7	141.9	3	positive	1146.7	117.2	3

Metabolisation with S9-mix

first experiment				second experiment			
conc.	revertants / plate			conc.	revertants / plate		
µl/plate	mean	SD	N	µl/plate	mean	SD	N
100	182.3	6.4	3	100	167.0	6.2	3
33	177.7	22.5	3	33	158.0	16.7	3
11	171.0	2.6	3	11	144.3	11.7	3
3.7	179.3	15.6	3	3.7	160.3	12.0	3
1.2	159.3	12.2	3	1.2	160.3	17.2	3
solvent	179.2	15.3	6	solvent	153.8	18.8	6
positive	523.3	56.9	3	positive	421.7	40.7	3

solvent: water

positive: without metabolisation: 4-Nitro-o-phenylene-diamine, 10 µg / plate
with metabolisation: 7,12-Dimethylbenz[a]anthracene, 5 µg / plate

: significantly higher than twice the amount of the control group

"UKRAIN 5 mg Ampullen": Salmonella Typhimurium Reverse Mutation Test

Table 2: Mean number of revertants per plate for strain TA98

Mean, standard deviations SD and number of plates N.

Without metabolisation

first experiment				second experiment			
conc.	revertants / plate			conc.	revertants / plate		
µl/plate	mean	SD	N	µl/plate	mean	SD	N
100	9.0	3.6	3	100	9.7	2.5	3
33	10.7	3.8	3	33	7.3	4.5	3
11	8.0	3.5	3	11	10.3	0.6	3
3.7	10.0	3.0	3	3.7	11.3	1.2	3
1.2	10.3	3.2	3	1.2	12.7	4.9	3
solvent	10.7	2.8	6	solvent	9.2	3.3	6
positive	393.3	63.5	3	positive	338.3	87.4	3

Metabolisation with S9-mix

first experiment				second experiment			
conc.	revertants / plate			conc.	revertants / plate		
µl/plate	mean	SD	N	µl/plate	mean	SD	N
100	19.7	5.5	3	100	19.7	4.0	3
33	16.7	3.2	3	33	23.7	3.2	3
11	17.3	2.1	3	11	15.3	2.5	3
3.7	16.0	6.6	3	3.7	14.0	4.6	3
1.2	15.0	3.0	3	1.2	19.3	1.2	3
solvent	15.7	5.0	6	solvent	15.2	3.5	6
positive	496.7	153.1	3	positive	596.7	70.9	3

solvent: water

positive: without metabolisation: 2-Nitrofluorene, 2 µg / plate
with metabolisation: 2-Amino-anthracene, 1 µg / plate

: significantly higher than twice the amount of the control group

"UKRAIN 5 mg Ampullen": Salmonella Typhimurium Reverse Mutation Test

Table 3: Mean number of revertants per plate for strain TA100

Mean, standard deviations SD and number of plates N.

Without metabolisation

first experiment				second experiment			
conc.	revertants / plate			conc.	revertants / plate		
µl/plate	mean	SD	N	µl/plate	mean	SD	N
100	113.7	3.1	3	100	107.3	11.9	3
33	95.3	15.0	3	33	102.7	4.5	3
11	109.7	10.7	3	11	105.3	3.8	3
3.7	101.0	4.4	3	3.7	100.3	11.7	3
1.2	103.0	5.3	3	1.2	105.7	4.7	3
solvent	106.2	9.6	6	solvent	97.3	12.4	6
positive	1040.0	20.0	3	positive	1090.0	111.4	3

Metabolisation with S9-mix

first experiment				second experiment			
conc.	revertants / plate			conc.	revertants / plate		
µl/plate	mean	SD	N	µl/plate	mean	SD	N
100	135.7	8.1	3	100	115.0	17.3	3
33	116.0	19.7	3	33	106.3	11.7	3
11	103.0	7.9	3	11	128.3	44.8	3
3.7	95.0	6.1	3	3.7	95.3	3.2	3
1.2	98.7	21.2	3	1.2	96.3	7.0	3
solvent	113.7	14.6	6	solvent	99.7	11.9	6
positive	2626.7	174.7	3	positive	3406.7	412.0	3

solvent: water

positive: without metabolisation: Sodium azide, 2 µg / plate

with metabolisation: 2-Amino-anthracene, 2 µg / plate

: significantly higher than twice the amount of the control group

"UKRAIN 5 mg Ampullen": Salmonella Typhimurium Reverse Mutation Test

Table 4: Mean number of revertants per plate for strain TA102

Mean, standard deviations SD and number of plates N.

Without metabolisation

first experiment				second experiment			
conc.	revertants / plate			conc.	revertants / plate		
µl/plate	mean	SD	N	µl/plate	mean	SD	N
100	216	5	3	100	250	18	3
33	222	27	3	33	255	6	3
11	223	12	3	11	251	27	3
3.7	228	26	3	3.7	241	13	3
1.2	207	11	3	1.2	251	6	3
solvent	224	16	6	solvent	266	27	6
positive	647	31	3	positive	717	25	3

Metabolisation with S9-mix

first experiment				second experiment			
conc.	revertants / plate			conc.	revertants / plate		
µl/plate	mean	SD	N	µl/plate	mean	SD	N
100	287	17	3	100	292	18	3
33	251	13	3	33	279	17	3
11	263	5	3	11	287	26	3
3.7	263	15	3	3.7	288	38	3
1.2	255	33	3	1.2	249	17	3
solvent	252	33	6	solvent	282	27	6
positive	687	70	3	positive	643	95	3

solvent: water

positive: without metabolisation: t-Butyl-hydroperoxide, 50 µg / plate

with metabolisation: 1,8-Dihydroxy-anthraquinone, 50 µg / plate

: significantly higher than twice the amount of the control group

"UKRAIN 5 mg Ampullen": Salmonella Typhimurium Reverse Mutation Test

Table 5: Mean number of revertants per plate for strain TA1535

Mean, standard deviations SD and number of plates N.

Without metabolisation

first experiment				second experiment			
conc.	revertants / plate			conc.	revertants / plate		
µl/plate	mean	SD	N	µl/plate	mean	SD	N
100	14.7	4.0	3	100	14.3	1.5	3
33	8.3	3.5	3	33	11.0	1.0	3
11	7.0	3.6	3	11	8.0	2.6	3
3.7	6.7	1.2	3	3.7	6.7	1.5	3
1.2	6.0	1.0	3	1.2	9.7	2.5	3
solvent	7.7	3.5	6	solvent	8.3	1.6	6
positive	270.0	25.0	3	positive	500.0	72.1	3

Metabolisation with S9-mix

first experiment				second experiment			
conc.	revertants / plate			conc.	revertants / plate		
µl/plate	mean	SD	N	µl/plate	mean	SD	N
100	19.0	2.6	3	100	15.7	2.1	3
33	14.7	2.1	3	33	12.7	1.2	3
11	8.7	3.2	3	11	7.3	2.3	3
3.7	8.0	4.6	3	3.7	9.3	4.2	3
1.2	9.0	1.0	3	1.2	8.0	1.0	3
solvent	10.0	2.8	6	solvent	9.0	2.9	6
positive	56.7	10.0	3	positive	50.7	12.7	3

solvent: water

positive: without metabolisation: Sodium azide, 1 µg / plate

with metabolisation: 2-Amino-anthracene, 2 µg / plate

: significantly higher than twice the amount of the control group

"UKRAIN 5 mg Ampullen": Salmonella Typhimurium Reverse Mutation Test

Table 6: Individual numbers of revertants per plates

Strain **TA97a**, experiment no. **1**

concentr. µl/plate	revertants without metabolisation			revertants with metabolisation		
100	149	153	143	185	175	187
33	145	146	136	156	201	176
11	159	150	149	172	168	173
3.7	153	140	170	196	177	165
1.2	154	173	172	170	146	162
solvent	176	150	177	159	186	176
solvent	149	175	149	204	170	180
positive	1140	1240	1420	570	460	540

Strain **TA97a**, experiment no. **2**

concentr. µl/plate	revertants without metabolisation			revertants with metabolisation		
100	141	145	146	174	165	162
33	133	126	140	173	140	161
11	142	145	112	134	157	142
3.7	161	158	134	172	161	148
1.2	132	126	118	174	166	141
solvent	152	111	156	173	169	141
solvent	130	137	128	160	157	123
positive	1100	1060	1280	450	375	440

solvent: water

positive: without metabolisation: 4-Nitro-o-phenylene-diamine, 10 µg / plate
with metabolisation: 7,12-Dimethylbenz[a]anthracene, 5 µg / plate

"UKRAIN 5 mg Ampullen": Salmonella Typhimurium Reverse Mutation Test

Table 6, cont.: Individual numbers of revertants per plates

Strain **TA98**, experiment no. **1**

concentr. µl/plate	revertants without metabolisation			revertants with metabolisation		
100	10	12	5	20	25	14
33	15	8	9	18	13	19
11	6	6	12	15	18	19
3.7	13	7	10	23	10	15
1.2	9	14	8	12	18	15
solvent	8	16	10	12	25	14
solvent	11	10	9	16	11	16
positive	320	430	430	590	580	320

Strain **TA98**, experiment no. **2**

concentr. µl/plate	revertants without metabolisation			revertants with metabolisation		
100	7	10	12	16	24	19
33	3	7	12	26	20	25
11	11	10	10	15	18	13
3.7	12	10	12	13	10	19
1.2	7	16	15	20	18	20
solvent	8	5	13	16	12	21
solvent	7	13	9	13	12	17
positive	265	315	435	660	610	520

solvent: water

positive: without metabolisation: 2-Nitrofluorene, 2 µg / plate
with metabolisation: 2-Amino-anthracene, 1 µg / plate

"UKRAIN 5 mg Ampullen": Salmonella Typhimurium Reverse Mutation Test

Table 6, cont.: Individual numbers of revertants per plates

Strain **TA100**, experiment no. **1**

concentr. µl/plate	revertants without metabolisation			revertants with metabolisation		
100	111	113	117	130	145	132
33	78	103	105	95	134	119
11	104	103	122	100	112	97
3.7	103	96	104	102	91	92
1.2	99	109	101	89	123	84
solvent	110	102	124	112	94	134
solvent	102	98	101	109	106	127
positive	1060	1020	1040	2480	2820	2580

strain **TA100**, experiment no. **2**

concentr. µl/plate	revertants without metabolisation			revertants with metabolisation		
100	99	102	121	95	124	126
33	103	98	107	96	104	119
11	101	108	107	180	100	105
3.7	98	90	113	99	94	93
1.2	102	111	104	103	97	89
solvent	87	92	82	90	109	89
solvent	102	107	114	89	116	105
positive	1070	1210	990	3720	2940	3560

solvent: water

positive: without metabolisation: Sodium azide, 2 µg / plate

with metabolisation: 2-Amino-anthracene, 2 µg / plate

"UKRAIN 5 mg Ampullen": Salmonella Typhimurium Reverse Mutation Test

Table 6, cont.: Individual numbers of revertants per plates

Strain **TA102**, experiment no. **1**

concentr. µl/plate	revertants without metabolisation			revertants with metabolisation		
100	222	213	213	288	303	270
33	243	192	231	237	255	261
11	234	225	210	258	264	267
3.7	216	210	258	246	267	276
1.2	204	198	219	222	288	255
solvent	231	198	216	249	243	285
solvent	222	234	243	252	288	198
positive	680	640	620	620	680	760

strain **TA102**, experiment no. **2**

concentr. µl/plate	revertants without metabolisation			revertants with metabolisation		
100	243	270	237	312	285	279
33	255	249	261	282	261	294
11	258	221	273	297	258	306
3.7	237	255	231	246	321	297
1.2	258	249	246	246	234	267
solvent	240	261	303	279	318	291
solvent	291	267	234	252	300	252
positive	740	720	690	610	570	750

solvent: water

positive: without metabolisation: t-Butyl-hydroperoxide, 50 µg / plate
with metabolisation: 1,8-Dihydroxy-anthraquinone, 50 µg / plate

"UKRAIN 5 mg Ampullen": Salmonella Typhimurium Reverse Mutation Test

Table 6, cont.: Individual numbers of revertants per plates

Strain **TA1535**, experiment no. **1**

concentr. µl/plate	revertants without metabolisation			revertants with metabolisation		
100	14	11	19	22	18	17
33	5	12	8	14	13	17
11	11	6	4	10	11	5
3.7	6	8	6	7	13	4
1.2	5	7	6	9	8	10
solvent	11	6	12	8	8	15
solvent	9	4	4	10	11	8
positive	270	295	245	56	67	47

Strain **TA1535**, experiment no. **2**

concentr. µl/plate	revertants without metabolisation			revertants with metabolisation		
100	13	16	14	15	18	14
33	12	10	11	14	12	12
11	7	6	11	6	10	6
3.7	7	5	8	8	14	6
1.2	7	10	12	8	9	7
solvent	10	8	9	7	6	13
solvent	7	6	10	12	9	7
positive	480	440	580	46	65	41

solvent: water

positive: without metabolisation: Sodium azide, 1 µg / plate

with metabolisation: 2-Amino-anthracene, 2 µg / plate

"UKRAIN 5 mg Ampullen": Salmonella Typhimurium Reverse Mutation Test

Table 7: Testing of the Salmonella strains

The tests were performed in November 1997. The strains were stored in liquid nitrogen since that time.

Genetic properties:

strain:	TA97a	TA98	TA100	TA102	TA1535
Sensitive against crystal violet	yes	yes	yes	yes	yes
Sensitive against ampicillin	no	no	no	no	yes
Sensitive against ampicillin/tetracycline	--	yes	--	no	--
Sensitive against UV	yes	yes	yes	no	yes

-- : not tested

Mutation frequency:
(historic data of mean revertants per petri dish, standard deviation SD and number of tests N)

	without metabolisation				with metabolisation (S9-mix)			
strain:	substance, concentration (µg/plate)	revertants/plate **mean**	SD	N	substance, concentration (µg/plate)	revertants/plate **mean**	SD	N
TA97a	Control	**137**	21	41	Control	**148**	18	41
	4-NoPD, 20 µg	**1644**	312	36	2-Aminoanthracene, 3µg	**1317**	199	36
	4-NoPD, 10 µg	**1112**	335	4	Benzo(a)pyrene, 2.5 µg	**385**	52	2
	-	-			DMBA, 5 µg	**571**	297	7
TA98	Control	**11.2**	3.0	41	Control	**17.6**	4.5	41
	2-Nitro-fluorene, 2 µg	**366**	207	5	2-Aminoanthracene, 3µg	**1216**	420	4
	4-NoPD, 5 µg	**765**	155	36	2-Aminoanthracene, 1µg	**303**	114	38
	-	-			Benzo(a)pyrene, 2.5 µg	**70**	27	4

4-NoPD = 4-Nitro-o-phenylene-diamine DMBA = 7,12-Dimethylbenz[a]anthracene

"UKRAIN 5 mg Ampullen": Salmonella Typhimurium Reverse Mutation Test

Table 7, cont.: Testing of the Salmonella strains

Mutation frequency:
(historic data of mean revertants per petri dish, standard deviation SD and number of tests N)

	without metabolisation				with metabolisation (S9-mix)			
strain:	substance, concentration (µg/plate)	revertants/plate **mean**	SD	N	substance, concentration (µg/plate)	revertants/plate **mean**	SD	N
TA100	Control	**103**	15	41	Control	**106**	16	41
	Na-Azide, 5 µg	**1548**	385	36	2-Aminoanthracene, 3µg	**2152**	764	36
	Na-Azide, 2 µg	**1228**	552	4	2-Aminoanthracene, 2µg	**2043**	1115	4
	2-Nitro-fluorene, 2 µg	**453**	0	1	Benzo(a)pyrene, 2.5 µg	**499**	185	4
TA102	Control	**254**	56	41	Control	**307**	60	41
	tBHPO, 50 µg	**667**	113	4	DHA, 50 µg	**893**	219	39
	-	-			Benzo(a)pyrene, 2.5 µg	**507**	163	3
TA1535	Control	**8.9**	1.7	41	Control	**10.0**	1.5	41
	Na-Azide, 5 µg	**1927**	653	4	2-Aminoanthracene, 3µg	**120**	30	30
	Na-Azide, 1 µg	**426**	92	38	2-Aminoanthracene, 2µg	**76**	31	9

DHA = 1,8-Dihydroxy-anthraquinone
4-NoPD = 4-Nitro-o-phenylene-diamine
tBHPO = tert.-Butyl-hydroperoxide
DMBA = 7,12-Dimethylbenz[a]anthracene

"UKRAIN 5 mg Ampullen": Salmonella Typhimurium Reverse Mutation Test

Appendix 1: Composition of media and solutions

Overnight-medium: 0.8 g nutrient broth (Difco, Detroit, USA, article No. 0003-01-6), 0.5 g NaCl p.a. in 100 ml H_2O, autoclaved.

Top-agar: 3 g agar-agar (Oxoid, Basingstoke, UK, agar no. 1, code L11), 2.5 g NaCl p.a. in 500 ml H_2O, autoclaved.

Minimal agar: 7.6 g KH_2PO_4 p.a., 27.2 g K_2HPO_4 p.a., 3.9 g $(NH_4)_2SO_4$ p.a. and 1 g Na_3-citrate p.a. in 800 ml H_2O. 0.4 g $MgSO_4 \times 7H_2O$ p.a. in 480 ml H_2O. 60 g agar-agar (Oxoid Agar no. 1, code L11) in 2.4 l H_2O. 20.5 g glucose (Merck, Darmstadt, D, article No. 8346) in 320 ml H_2O. Autoclaved separately and combined thereafter. Immediately before use 40 ml of histidine-biotin solution are added per litre of agar.

Histidine-biotin solution: 13 mg histidine-hydrochloride- monohydrate (Merck, article No. 4350) and 15 mg biotin (Merck, article No. 24514) per 100 ml of H_2O, sterile filtered.

S9-mix: Each ml of microsomal fraction is combined with the following cofactor solution.

Cofactor solution: 13.2 ml of 0.2 mol/l Na_2HPO_4, 2.6 ml of H_2O, 0.3 ml of 1.65 mol/l KCl, 0.3 ml of 0.4 mol/l $MgCl_2$, 31 mg of d-glucose-6-phosphate.Na_2 (Sigma, article G-7250) and 56 mg of NADP (Sigma, article N-0505).

Phosphate buffered saline (PBS): 8 g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4 \times 2\ H_2O$, 0.2 g KH_2PO_4 in 1000 ml H_2O.

"UKRAIN 5 mg Ampullen": Salmonella Typhimurium Reverse Mutation Test

Appendix 2: Certificate of analysis of the test substance

NO 6

Nowicky Pharma
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Analysenzertifikat: Ukrain 5mg Ampullen

Chargennumer: 544324 **Hergestellt: Oktober 1996**

Parameter	Sollwert	Istwert
1. Allgemeine Merkmale		
Aussehen	klare, hellgelbe wäßrige Lösung, nahezu geruchlos	entspricht
2. Identität		
DC-Analyse	vergleichbare DC-Spots wie Referenzstandard	entspricht
3. Verunreinigungen		
Nicht umgesetzte Alkaloide (Chelidonin und Sanguinarin)	max. 150 µg/ml	127,5 µg/ml
Schwermetalle (ber. als Pb)	max. 10 ppm	< 1 ppm
4. Gehalt		
Ukrain, bezogen auf Referenzstandard	90-110%	95,9%
5. Andere Prüfungen		
PH-Wert	3,0 – 5,0	3,49
Dichte	0,9980 – 1,0050	0,9986
Entnehmbares Volumen	5,10 – 5,40 ml	5,15 ml
Sterilität	steril nach Pharm EU	steril
Pyrogentest	pyrogenfrei nach Pharm EU	apyrogen

Freigegeben am 16. 1. 1997
Gesperrt am .....

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