

BIOASSAY SYSTEMS CORPORATION
225 Wildwood Avenue
Woburn, MA 01801

FINAL REPORT

SALMONELLA/MICROSOME MUTAGENESIS ASSAY

ON

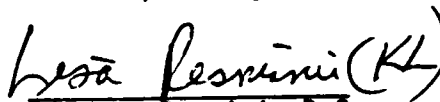
SC-19129

BSC Project Number: 12157

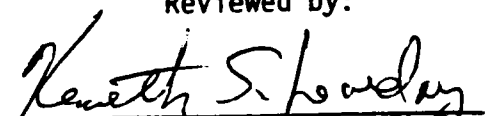
Prepared for:

G. D. Searle & Co.
4901 Searle Parkway
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Prepared by:


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March 20, 1985

Date

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TITLE: Salmonella/microsome Mutagenesis Assay on SC-19129

Author: Lisa M. Resmini, B.S.
(Bioassay Systems Corporation, Woburn, Massachusetts)

Study Number: S.A. 2462

Date: March 20, 1985 Type of Report: Final

Summary:

SC-19129 was investigated for the potential to induce mutations in Salmonella typhimurium strains TA1535, TA1537, TA1538, TA98 and TA100 in the presence and absence of a rat liver homogenate metabolic activation system. The intended test concentrations were 10, 7.5, 5.0, 1.5, and 0.5 mg/plate. The actual test concentrations based on analysis of the stock solution were 85.1% of the target concentrations. No evidence of a positive mutagenic response was seen. The responses obtained from the negative and positive controls demonstrated that the test system was capable of detecting chemical mutagens.

These results lead to the conclusion that SC-19129 is non-mutagenic under the conditions of the Salmonella/microsome mutagenesis assay.

1.0 INTRODUCTION

1.1 Objective of the Study

The purpose of this study was to investigate the potential of SC-19129 to induce mutations in Salmonella typhimurium, using an in vitro mutagenesis assay. Aliquot 84-1226A of the sample was received for testing on October 19, 1984 and aliquot 84-1226B was received for testing on October 31, 1984. The range finding assay was initiated December 5, 1984. Mutagenesis testing was initiated December 10, 1984 and completed on January 17, 1985. All assays were conducted according to FDA Good Laboratory Practice Regulations: 21 CFR 58.1-58.219, 1979.

1.2 Principles of the Assay

The simplest, least expensive, and most widely used short-term assay for the detection of chemical mutagens is the Salmonella/microsome mutagenesis assay. The Salmonella test measures the ability of a test substance to induce mutations in the histidine biosynthetic pathway of Salmonella typhimurium. The bacterial strains used in the test are unable to grow in the absence of histidine (his^-). In the presence of mutagenic substances, some bacteria undergo mutations so that they no longer require histidine for growth (his^+). The strains have been specially constructed to detect as many classes of mutagens as possible. For example, the cell wall of the bacteria has been modified to permit the entry of large molecules and the excision repair system has been deleted. In addition, a plasmid has been introduced into two of the strains (TA98 and TA100) to increase the sensitivity of these strains to some classes of mutagens. Finally, each strain has been constructed to detect mutagens which cause either point mutations (substitution of one base for another in the DNA) or frameshift mutations (addition or deletion of one or more bases). The use of a rat liver homogenate (also called S-9) provides the enzymes found in most mammals needed to metabolize many potential mutagens to their reactive forms.

2.0 MATERIALS

2.1 Test Substance

Name:	SC-19129
BSC No.:	84-1226A and 84-1226B
Lot/Batch No.:	84K-047-101
Physical State:	Solid; powder
Color:	White
Density:	N/A
Purity:	greater than 99%
Composition:	Determined by Sponsor
Stability:	Determined by Sponsor
Stability of Formulations:	Determined by Sponsor
Solubility:	Dimethylsulfoxide (greater than 100 mg/ml)
Storage Conditions:	Ambient, protect from light
Safety Precautions:	Routine

The test sample was assayed in solution using dimethylsulfoxide as a vehicle. A stock solution of 100 mg/ml was used for activated and nonactivated assays. All further dilutions were made fresh in dimethylsulfoxide. Concentration analysis (Appendix B) confirmed the stock solution's concentration (85.1%). Aliquot 84-1226A was used for the range finding assay; aliquot 84-1226B was used for mutagenesis testing.

The Sponsor assumed responsibility for determining the identity, strength, purity, composition and stability of the test sample and the stability of the formulations.

2.2 Negative Control Substance

Name:	Dimethylsulfoxide (DMSO)
Supplier:	Baker Chemical Co.
Lot/Batch No.:	144601 and 327701
Physical State:	liquid
Color:	clear
Purity:	reagent grade
Composition:	on file with manufacturer
Stability:	indefinite
Storage Conditions:	room temperature
Safety Precautions:	avoid topical and respiratory contact

2.3 Positive Control Substances

	Nonactivated	Nonactivated	Nonactivated
Name:	9-aminoacridine	sodium azide	2-nitrofluorene
Supplier:	Sigma	Sigma	Aldrich
Lot/Batch No.:	117C-0119	92C-2900	112967
Physical State:	solid	solid	solid
Color:	yellow	white	white

Purity:	90%	99%	98%
Solubility:	DMSO	DMSO, water	DMSO
Stability:	indefinite	indefinite	indefinite
Stability of formulations:	1 yr.at -20°C	1 yr.at -20°C	1 yr.at -20°C
Storage Conditions:(solid)	4°C	room temp.	room temp.
(solutions)	-20°C	-20°C	-20°C
Composition:	on file with manufacturer		
Safety Precautions:	avoid topical and respiratory contact		
Stock Concentration:	1 mg/ml	25 ug/ml	100 ug/ml

Stock solutions of the nonactivated positive control agents were prepared in DMSO (Baker 052348) and stored at -20°C. Aliquots of the stock solutions are added to the plates.

Activated

Name:	2-anthramine
Supplier:	Sigma
Lot/Batch No.:	110F-0600
Physical State:	solid
Color:	gold
Purity:	99%
Composition:	on file with with manufacturer
Solubility:	DMSO
Stability:	indefinite
Stability of formulations:	at least 24 hours at room temperature
Storage Conditions: (solid)	4°C
(solution)	prepared prior to use
Safety Precautions:	avoid topical and respiratory contact
Stock Concentration:	10 ug/ml

The stock solution of the activated positive control agent was prepared in DMSO (Baker 052348) prior to use. Aliquots of the stock solution are added to the plates.

2.4 Bacterial Culture

The Salmonella typhimurium strains TA98, TA100, TA1538, TA1537, and TA1535 used in this assay were obtained from Dr. Bruce Ames at the University of California, Berkeley, CA.

Master vials are stored at -80°C (10% DMSO added), and working plates are prepared monthly and stored at 4°C. Daily cultures are grown overnight in Oxoid nutrient broth from colonies on working plates.

2.5 Microsomal Activation System

An S9 microsome fraction prepared from the liver of Aroclor 1254 induced (500 mg/kg) Sprague-Dawley rats was used in the activated

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assay. The S9 mix consisted of 3% rat liver S9 fraction in a cofactor mixture of 8mM MgCl₂, 33mM KCl, 5mM glucose-6-phosphate, 4mM NADP and 100 mM Na₂ HPO₄, pH 7.4. For the test 0.5 ml of S9 mix was added to the appropriate plates. The S9 mixture was prepared fresh and maintained at 4°C until used.

The following S9 fraction was used in the study:

Source: Microbiological Associates
Lot/Batch No.: R192
Storage Conditions: -80°C

2.6 Identification of Test System

All the experimental vessels (plates and dilution tubes) were labeled with the last three digits of the project number, strain number (where appropriate) and a code number. The key to the code numbers are given in the raw data sheets.

3.0 EXPERIMENTAL DESIGN:

3.1 Rangefinding Assay

For the rangefinding assay the method used for the mutagenesis assay was followed using Salmonella strain TA100 and nonactivated conditions. Duplicate plates were employed. Toxicity was evaluated on the basis of the number of revertants per plate relative to the negative control and the condition of the bacterial background lawn. Sample sterility was also assessed. The concentrations for the mutagenesis assay were chosen on the basis of the results from the range-finding assay.

3.2 Mutagenesis Assay

Five Salmonella strains were used: TA98, TA100, TA1535, TA1537 and TA1538. Bacterial strains were checked the day of the assay to ensure that all five strains were sensitive to crystal violet (rfa mutation) and that TA98 and TA100 contained the R-factor (ampicillin resistance).

The following components were added sequentially to 2 ml aliquots of molten top agar containing 50 uM biotin and 50 uM histidine: 0.1 ml of test sample or control concentration, 0.1 ml overnight bacterial culture, and, for the activated portion of the assay, 0.5 ml S9 mix. The contents of the tubes were mixed and plated on minimal medium bottom agar petri dishes. After 2 days of incubation at 37°C, plates were scored for visible colonies.

4.0 RESULTS:

In the range-finding assay, the sample was tested from 20 mg/plate to 0.02 mg/plate. There was no evidence of toxicity of the test article (Table 1). Based on this information, an initial

mutagenesis assay was performed using 20 mg/plate as the top concentration. Concentration analysis for this assay could not be performed within the required three days. The results for this assay are, therefore, considered invalid and are not reported.

The Sponsor then requested the sample to be tested using 10 mg/plate as the highest concentration, since solubility problems were noted in the stocks at 200 mg/ml. The stock solution used in this assay was maintained at 37°C until dilutions were made. The solution remained clear. The results of the mutagenesis assay are shown in Table 2. No evidence of a positive response was observed for any strain. No toxicity was seen. The known mutagens used as positive controls gave normal mutagenic responses. The negative control values were within historical limits.

5.0 CONCLUSION:

SC-19129 did not induce increases in mutations in Salmonella typhimurium. Therefore, under the conditions of the Salmonella/microsome Mutagenesis Assay, the compound can be considered non-mutagenic.

Table 1
Preliminary Toxicity Assay on SC-19129 using Strain TA100

Concentration of Sample and Control	Avg. # Colonies*	Observations
20 mg/plate	93	Normal Lawn
6 mg/plate	93	Normal Lawn
2 mg/plate	118	Normal Lawn
0.6 mg/plate	109	Normal Lawn
0.2 mg/plate	81	Normal Lawn
0.06 mg/plate	94	Normal Lawn
0.02 mg/plate	0 ^a	Normal Lawn
Negative Control: 100 ul/plate DMSO ^b	93	Normal Lawn

*Duplicate plates employed

^aBacteria were added to this plate, lawn was normal. There is no explanation for lack of revertants on this plate. However, since this only occurred at the low dose it can be assumed that it was not a test article related effect.

^bJ.T. Baker Lot No. 327701

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Table 2
Quantitative Mutagenesis Assay on SC-19129

Concentration of Sample and Controls	Metabolic Activation	Salmonella strains*				
		TA98	TA100	TA1535	TA1537	TA1538
10 mg/plate	+	24	100	18	6	17
	-	17	96	15	6	9
7.5 mg/plate	+	26	93	14	5	18
	-	11	103	20	6	11
5.0 mg/plate	+	21	85	14	6	21
	-	15	118	21	5	10
1.5 mg/plate	+	24	91	9	7	17
	-	18	87	20	4	13
0.5 mg/plate	+	24	102	21	4	22
	-	15	97	18	6	12
Negative Control: 100 ul DMSO/plate ^b	+	27	92	17	5	23
	-	19	116	24	6	8
Sodium azide 2.5 ug/plate Strains TA100, TA1535	-		704	561		
2-nitrofluorene 10 ug/plate Strains TA98, TA1538	-	301				846
9-aminoacridine 100ug/plate Strain TA1537	-				832	
2-anthramine 1.0 ug/plate All strains	+	2161	1118 ^a	161	375	1962

*Figures represent the number of his⁺ revertants per plate and are the average of three replicate assay plates.

^aValues are: 2239, 1009, 107

^bJ.T. Baker Lot No. 144601

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Quality Assurance Report

Study Title: Salmonella/microsome Mutagenesis Assay on
SC-19129
Sponsor: G.D. Searle & Co.
BSC Project Number: 12157
BSC Sample Number: 84-1226A (Range finder) and 84-1226B
(Mutagenesis Assay)

<u>Date(s) of Inspection</u>	<u>Date Findings Reported to Study Director</u>	<u>Date Findings Reported to Management</u>
10/31/84	10/31/84	11/5/84
12/5/84	12/5/84	12/10/84
1/15/85	1/16/85	1/21/85
2/4/85	2/4/85	2/6/85
3/15/85	3/15/85	3/20/85

Date: 3/20/85

Quality Assurance Officer: Susan M. O'Connor

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APPENDIX A

Appendix A

1. Supervisory Personnel

Kenneth S. Loveday, Ph.D., Director of Genetic Toxicology
Lisa M. Resmini, B.S., Study Director
Susan M. O'Connor, B.S., Manager, Quality Assurance

2. Storage Location Information

Raw Data: BSC Archives
Final Report: BSC Archives

APPENDIX B

REPORT OF ANALYSIS

BSC PROJECT NO(s): 12157

DATE OF ANALYSIS: 1/15-18/85

SPONSOR: G. D. Searle

TEST SAMPLE IDENTIFICATION

Sponsor Identification: SC19129 B-APM

BSC Sample No.: 84-1226B

CHEMICAL VEHICLE: Dimethyl sulfoxide (DMSO)
Source: Baker Chemical Co.
Lot No.: 144001

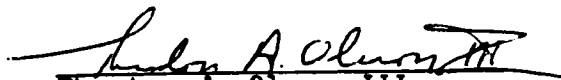
METHOD OF ANALYSIS: BSC Standard Operating Procedure # 103-146

SUMMARY OF RESULTS:

1. System Suitability: Mean Standard Peak Area: 74.3458
Relative Standard Deviation (RSD): 2.3%
Number of Injections: 6
2. Standard Check - Percent of Theory: 100.7%
3. Control Sample - Percent Recovery: 83.4%
4. Test Samples - Concentrations Measured/% Recovery
 - a. Formulation Lot No. 1-15-85(b): 85.14 mg/ml
85.1% Recovery

REVIEW OF ANALYSIS

The results reported above have been reviewed and found to accurately represent the data collected during this analysis. Please see "Comments" section for further discussion.


Theodore A. Olsson III
Manager, Chemistry

Date 3-18-85

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REPORT OF ANALYSIS

BSC PROJECT NO(s): 12157

DATE OF ANALYSIS: 1/15-18/85

SUMMARY OF DATA

Sample/Standard Identification	Preparation* of Sample/Standard	Injection No.	Peak Area	Mean Peak Area
Standard	0.1000g to 100 ml with mobile phase	1(a)	74.9491	74.3458 (2.3% RSD)
		2(a)	74.8979	
		3(a)	75.5072	
		4(a)	70.9273	
		5(a)	74.9926	
		6(a)	74.8006	
		13(b)	71.3344	74.5508
		18(b)	77.6422	
		23(b)	74.2169	
		28(b)	73.3440	
		33(b)	76.2163	
Standard Check	0.1000g to 100 ml with mobile phase	7(b)	60.5516**	74.0518
		21(b)	74.3782	
		22(b)	73.7254	
Control Sample	0.0999g +1ml DMSO to 100 ml with mobile phase	9(b)	63.1654	62.1459
		10(b)	55.3625	
		24(b)	73.0419	
		25(b)	57.0137	
Test Sample 2 (Form Lot # 1-15-85b)	1 ml to 10.0 ml with mobile phase	14(b)	63.3446	63.4700
		15(b)	54.2924	
		29(b)	70.4614	
		30(b)	65.7817	

* All standards and samples were diluted 1.0 ml to 10.0 ml with mobile phase after the preparation noted above, with the exception of Test Sample #2. Due to technician error this sample was diluted 1.0 ml sample to 10ml with mobile phase then 1.0 ml of this dilution to 100 ml with mobile phase.

** Using "Dixon's Criteria" this value (at the 5% level) may be considered an outlier and therefore is not used. Evidence of misintegration was also observed.

(a) System suitability check-prior to analysis

(b) Sample and standard chromatographic runs over the period of 1/16-18/85, for the analysis of the test samples.

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REPORT OF ANALYSIS

BSC PROJECT NO(s).: 12157

DATE OF ANALYSIS: 1/15-18/85

SUMMARY OF CALCULATIONS:

1. Standard Check - Percent of Theory

$$\begin{aligned} \% \text{ STD} &= \frac{R \text{ STD}}{R \text{ STDCK}} \times \frac{W \text{ STD CK}}{W \text{ STD}} \times 100\% \\ &= \frac{74.5508}{74.0518} \times \frac{100.0 \text{ mg}}{100.0 \text{ mg}} \times 100 \\ &= 100.7\% \end{aligned}$$

2. Control Sample - Percent Recovery

$$\begin{aligned} \% \text{ Recovery} &= \frac{R \text{ CS}}{R \text{ STD}} \times \frac{\text{Conc. STD}}{\text{Conc. CS}} \times 100\% \\ &= \frac{62.1459}{74.5508} \times \frac{1.000 \text{ mg/ml}}{0.999 \text{ mg/ml}} \times 100\% \\ &= 83.4\% \end{aligned}$$

3. Concentrations of Test Sample

$$\text{Conc. Test Sample (SC19129 mg/ml DMSO)} = \frac{R \text{X}}{R \text{STD}} \times \text{Conc. STD} \times \text{dilution factor}$$

a) Test Sample Form. Lot No.1-15-85b used for in vitro study - Ames Test

$$\begin{aligned} \text{Conc. Test Sample} &= \frac{63.4700}{74.5508} \times 1.000 \text{ mg/ml} \times 100 \\ &= 85.14 \text{ mg/ml} \end{aligned}$$

$$\begin{aligned} \text{Expected Conc.} &= 100 \text{ mg/ml} \\ \% \text{ Recovery} &= 85.1\% \end{aligned}$$

COMMENTS

Due to technical problems, the analysis was conducted from 1/15-18/85.

Note: R STD = mean peak height or area for Standard
 R STD CK = mean peak height or area for Standard Check
 W STD = weight of test sample in Standard (mg)
 W STD CK = weight of test sample in Standard Check (mg)
 R CS = mean peak height or area for Control Sample
 Conc STD = concentration of test sample in Standard (mg/ml equivalent DMSO)
 Conc CS = concentration of test sample in Control Sample (mg/ml DMSO)

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1.0 Study Title

Salmonella/microsome Mutagenesis Assay on SC-19129

2.0 Purpose of Study

The purpose of this study is to investigate the potential of the test substance(s) to induce mutations in Salmonella typhimurium, using an in vitro mutagenesis assay.

3.0 Management of Study

3.1 Sponsor's Name and Address:

G.D. Searle & Co.
4901 Searle Parkway
Skokie, IL 60077

3.2 Sponsor's Study Coordinator: Charles E. Piper, Ph.D.

3.3 Testing Laboratory's Name Address:

Bioassay Systems Corporation
225 Wildwood Avenue
Woburn, MA 01801

BSC Project Number: 12157

3.4 Supervisory Personnel:

Director of Genetic Toxicology: Kenneth S. Loveday, Ph.D.

Study Director: Lisa M. Resmini, B.S.

3.5 Manager, Quality Assurance: Susan O'Connor, B.S.

3.6 Proposed Study Schedule:

Test Substance Received:	10/19/84
Technical Work Initiated:	11/5/84
Technical Work Completed:	12/7/84

4.0 Test Material Data

4.1 Test Sample Characterization:

- 4.1.1 Name: SC-19129
- 4.1.2 BSC No.: To be specified in final report
- 4.1.3 Lot No.: 84K-047-101
- 4.1.4 Physical State: Solid; powder
- 4.1.5 Color: White
- 4.1.6 Density: Not applicable
- 4.1.7 Purity: > 99%
- 4.1.8 Composition: To be determined by Sponsor
- 4.1.9 Stability of Bulk Compound: To be determined by Sponsor
- 4.1.10 Stability of Formulations: To be determined by Sponsor
- 4.1.11 Solubility: Dimethylsulfoxide (100 mg/ml)
- 4.1.12 Storage Conditions: Ambient, protect from light
- 4.1.13 Safety Precautions: Routine

The Sponsor will determine the identity, strength, purity, composition and stability of the test article. Bioassay Systems will determine concentration of the test article in carrier. Sponsor will analyze the test article for identity (at a minimum) upon return from the testing laboratory.

4.2 Positive Control Material Characterization (Nonactivated Assay)

4.2.1 Name: 9-aminoacridine sodium azide 2-nitrofluorene

4.2.2 Supplier: To be specified in final report

4.2.3 Lot/Batch No.: To be specified in final report

4.2.4 Physical State: solid solid solid

4.2.5 Color: yellow white white

4.2.6 Purity: 90% 99% 98%

4.2.7 Composition: on file with Manufacturer

4.2.8 Stability: (solid) indefinite indefinite indefinite
(solution) one year at one year at one year at
-20°C -20°C -20°C

4.2.8 Solubility: DMSO DMSO, water DMSO

4.2.9 Storage Conditions: (solid) 4°C RT RT
(solutions) -20°C -20°C -20°C

4.2.10 Safety Precautions: avoid topical and respiratory contact

4.3 Positive Control Material Characterization (Activated Assay)

4.3.1 Name 2-anthramine

4.3.2 Lot/Batch No.: To be specified in final report

4.3.3 Supplier: To be specified in final report

4.3.4 Physical State: solid

4.3.5 Color: gold

4.3.6 Purity: 99%

4.3.7 Composition: on file with manufacturer

4.3.8 Stability: (solid) indefinite
(solution) one year at -20°C

4.3.9 Solubility: DMSO

4.3.10 Storage Conditions: (solid) 4°C*
(solution) -20°C

4.3.11 Safety Precautions: avoid topical and respiratory contact

4.4 Negative Control Material Characterization

4.4.1 Name: Dimethylsulfoxide (DMSO)

4.4.2 Supplier: J.T. Baker Chemical Co.

4.4.3 Lot/Batch No.: To be specified in final report

4.4.4 Physical State: liquid

4.4.5 Color: clear

4.4.6 Purity: reagent grade

4.4.7 Composition: on file with manufacturer

4.4.8 Stability: indefinite

4.4.9 Storage Conditions: room temperature

4.4.10 Safety Precautions: avoid topical and respiratory contact

* dessicate

Test System Specifications

- 5.1 Species: Salmonella typhimurium strains TA98, TA100, TA1535, TA1537, TA1538 were obtained from Dr. Bruce N. Ames, Univ. of California, Berkeley, CA.
- 5.2 Storage: Master vials are stored at -80 C (10% DMSO added). Working plates are prepared monthly and stored at 4°C. Daily cultures are grown overnight from colonies on working plates. Routine culture methods, preparation of media, reagents, S9 mix and quality control measures are detailed in Bioassay Systems Corporation's standard operating procedures.
- 5.3 Justification: Large existing data base, assay procedures are well-validated.
- 5.4 Identification of Test System: All the experimental vessels (plates and dilution tubes) will be labelled with the last three digits of the project no. and a code no. The key to the code will be given in the raw data sheets.
- 5.5 Culture Media:
- Working culture plates consist of bottom agar supplemented with 40% glucose and 50x VB salts and top agar supplemented with NaCl, histidine and biotin.

6.0 Experimental Design

The following outline briefly summarizes the experimental design.

- A. Strain Marker Verification
- B. Positive Control Preparation
- C. Range-finding Assay (without S9)
- D. Mutagenesis Assay (with and without S9)

6.1 Strain Marker Verification

Identification of the strains used in the mutagenesis assay is accomplished by streaking each strain on a nutrient agar plate. An ampicillin disc and a crystal violet disc are placed on opposite ends of each streak. Strains with rfa mutations (TA 98, TA 100, TA 1535, TA 1537 and TA 1538) will not grow in the presence of crystal violet; strains with R-factor, plasmid pKM 101, (TA 98, TA 100) grow in the presence of ampicillin.

6.2 Positive Control Preparation

All positive controls are dissolved in DMSO. Three positive controls are used in the nonactivated mutagenesis assay:

Name	Stock Sol'n Conc	Conc/plate	Strain
9-aminoacridine	1 mg/ml	100 ug/plate	TA1537
2-nitrofluorene	100 ug/ml	10 ug/plate	TA98, TA1538
sodium azide	25 ug/ml	2.5 ug/plate	TA100, TA1535

One positive control is used in the activated assay.

2-anthramine	10 ug/ml	1 ug/plate	All strains
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6.3 Range-finding Assay

In order to determine the amount of test sample to be tested in the mutagenesis assay, a range-finding assay is performed using strain TA100. Methods will follow those of the mutagenesis assay. The highest concentration tested will be the limit of solubility for solids or 10 mg/plate. Subsequent dilutions will be by half-logs (for example, ratios of 10, 3, 1 etc.) until 5-10 concentrations have been tested. Aliquots of 100 ul of test or control sample are added to each plate. All test points are performed in duplicate. The range-finding assay is performed without metabolic activation. Sample sterility will also be assessed.

Concentrations to be tested and the method of formulation in the mutagenesis assay will be documented in a protocol amendment.

6.4 Mutagenesis Assay:

Bacterial cultures are grown overnight in a shaking water bath, monitored with a klett meter and stored on ice. A minimum of five test sample concentrations and positive and negative controls are tested in triplicate. The highest concentration tested is that which resulted in a decrease in the number of revertant bacteria. Survival in the lowest concentration should be approximately that of the untreated controls.

Exposure to the Test Compound:

The test sample or control substance, 100 ul of overnight bacterial culture and 0.5 ml S9 mix (where appropriate) are added in that order to 2 ml top agar maintained in a tube at 45-50°C. The contents of each tube are mixed and poured onto a bottom agar plate. Plates are incubated for 48-72 hours at 37°C. The number of colonies on each plate is counted using an automatic colony counter.

6.5 Data Evaluation

The mean number of revertants per plate is calculated for each concentration and strain. A test result is considered positive, if for any strain, a significant increase over the negative control in the number of revertants per plate is observed which is concentration-dependent. A significant increase is a two-fold increase when the background is 50 revertants/plate or greater; a three fold increase when the background is between 10 and 49 revertants/plate, and a four-fold increase when the background is less than 10 revertants per plate. Results for a strain will be rejected if the positive control does not yield a mutagenic response or the negative control falls outside the 99% confidence limit of the historical background.

7.0 Report:

At the termination of the study, a draft and a final report will be prepared describing or containing: purpose of study, experimental design, sample properties and preparation, tabular and textual presentation of data, statistical analysis of data where appropriate, summary of results, conclusions, and quality assurance information (final report only).

8.0 Records to be Retained:

All original data and a copy of the final report will be retained for not less than five years after completion of the study and stored in the Bioassay Systems archives. This material will be made available for inspection upon request or by permission of authorized representatives of the sponsor. The sponsor will be notified before final disposition of these items. The test sample will be returned to the Sponsor upon completion of testing.

9.0 Quality Assurance:

This study will be monitored under provisions of the BSC Quality Assurance Program and the final report will be reviewed by BSC Quality Assurance personnel. This study will be conducted in accordance with FDA Good Laboratory Practice Regulations (21 CFR 58.1-58.219, 1979).

10.0 Protocol Changes:

All changes in or revisions of an approved protocol and the reasons therefor shall be documented, signed by the study director, the Study Coordinator and a BSC Quality Assurance officer, dated and maintained with the protocol.

11.0 Protocol Approval

11.1. Bioassay Systems Corporation

By: Lisa M. ReeminiTitle: Study DirectorDate: 10/30/84

11.2 Bioassay Systems Corporation Quality Assurance Unit

Content Approval

By: Susan M. O'ConnorTitle: Mgr., Quality AssuranceDate: 10/31/84

11.3 G.D. Searle & Co.

Charles E. PiperCharles E. Piper, Ph.D.
Diplomate, A.B.T.
Study Coordinator
Product Safety Assessment11/2/84
DateFrank N. KotsonisFrank N. Kotsonis, Ph.D.
Diplomate, A.B.T.
Director, Toxicology
Product Safety Assessment11/2/84
DateFred E. KohnFred E. Kohn, Ph.D.
Senior Director
Product Safety Assessment11/2/84
Date

Bioassay Systems Corporation

Protocol Amendment Form

Sponsor Name: G.D. Searle
BSC Project Number: 12157 BSC Sample Number: 84-1226
Study Title: Ames Test
Protocol Amendment Number: 1

Section 3.6

The date of initiation of the Range Finding Assay is December 5, 1984

	Date
Study Director Signature: <u>Lisa M. Benmini</u>	<u>12/4/84</u>
BSC Quality Assurance Officer Signature: <u>Nancy Gervino</u>	<u>12/4/84</u>
Sponsor Representative Signature (if applicable): <u>Charles E. Tien</u>	<u>12/11/84</u>
Telephone Authorization of Sponsor (if applicable): _____	

Bioassay Systems CorporationProtocol Amendment Form

Sponsor Name: G.D. Searle
BSC Project Number: 12157 BSC Sample Number: 81-1226
Study Title: Ames Test
Protocol Amendment Number: 2

- Section 3.6
The initiation date of the mutagenesis assay is December 10, 1984

Section 4.3.10
The 2 anthramine solution used in the assay will be made prior to use. It will not be stored at -20°C

Section 6.3
The concentrations to be tested in the mutagenesis assay are:
20 mg/plate
15 mg/plate
10 mg/plate
3 mg/plate
1 mg/plate
The vehicle will be DMSO

Section 6.4
The final sentence in this section should be amended to read as follows: "The number of colonies on each plate is counted according to BSC's counting S.O.P. Counting Procedures: Ames Plates and UDS slides."

	Date
Study Director Signature: <u>Lisa M. Benmisi</u>	<u>12/7/84</u>
BSC Quality Assurance Officer Signature: <u>Lisa M. Benmisi</u>	<u>12/7/84</u>
Sponsor Representative Signature (if applicable): <u>Charles E. Piper</u>	<u>12/11/84</u>
Telephone Authorization of Sponsor (if applicable): _____	

Bioassay Systems Corporation

Protocol Amendment Form

Sponsor Name: G.D. Searle
BSC Project Number: 12157 BSC Sample Number: 84-1226
Study Title: Ames Test
Protocol Amendment Number: 3

Section 4.1.11
The solubility of the test article in DMSO is greater than 100 mg/ml

Section 4.0
Due to technical problems, the test article ^{concentration} analysis for the Ames test will not be performed. The Study Coordinator was informed of this problem and authorized the Ames test to be conducted without concentration analysis.

Study Director Signature: Lina M. Benvenuti Date ^{as signed} 12/17/84 ^{working date} 12/12/84
BSC Quality Assurance Officer Signature: Nancy Herring 12/12/84
Sponsor Representative Signature (if applicable): Charles S. Piper 12/14/84
Telephone Authorization of Sponsor (if applicable): Dr. Charles Piper 12/10/84

Bioassay Systems Corporation

Protocol Amendment Form

Sponsor Name: G.D. Searle
BSC Project Number: 12157 BSC Sample Number: 81-1226
Study Title: Ames Test
Protocol Amendment Number: 4

Due to the difficulty involved in preparing the sample at 200mg/ml and the fact that no concentration analysis data are available because of equipment failure, the results of the mutagenesis assay conducted on 12/10/84 are considered invalid and will not be reported. The entire mutagenesis assay will be repeated with concentration analysis.

Section 3.6
The initiation date of the repeat mutagenesis assay is January 15, 1985

Section 6.3
Concentrations to be tested are: 10 mg/plate
7.5 mg/plate
5 mg/plate
1.5 mg/plate
0.5 mg/plate The vehicle will be DMSO

Protocol Amendment #1, Section 4.3.10 of Amendment #2, Section 6.4 of Amendment #2, and Section 4.1.11 of Amendment #3 will apply to the test⁴ conducted on 1/15/85.

A LR 1/19/85 Misentry

	Date
Study Director Signature: <u>Lina M. Buxton</u>	<u>1/19/85</u>
BSC Quality Assurance Officer Signature: <u>James M. O. Camp</u>	<u>1/9/85</u>
Sponsor Representative Signature (if applicable): <u>Charles E. Piper</u>	<u>1/15/85</u>
Telephone Authorization of Sponsor (if applicable): <u>Dr. Charles Piper</u>	<u>1/8/85</u>

Bioassay Systems Corporation

Protocol Amendment Form

Sponsor Name: G.D. Searle
BSC Project Number: 12107 BSC Sample Number: 84-1226
Study Title: Ames Test
Protocol Amendment Number: 5

Section 4.0 Test Material Data

The second sentence of this section should be amended as follows:

Bioassay Systems will determine concentration of the test article in carrier according to Bioassay Systems Corporation S.O.P. series 103, number 146.

Study Director Signature: <u>Lisa M. Benmini</u>	Date: <u>1/11/85</u>
BSC Quality Assurance Officer Signature: <u>Lisa M. Benmini</u>	<u>1/14/85</u>
Sponsor Representative Signature (if applicable): <u>Charles E. Piper</u>	<u>1/17/85</u>
Telephone Authorization of Sponsor (if applicable): _____	

Bioassay Systems Corporation

Protocol Amendment Form

Sponsor Name: GDS
BSC Project Number: 12157 BSC Sample Number: 84-1226
Study Title: Ames Test
Protocol Amendment Number: 6

Section 3.6

The completion date for the Ames Mutagenesis Assay is January 17, 1985. This was the day of colony counting.

Study Director Signature:	<u>Lisa M. Rimini</u>	Date	<u>1/23/85</u>
BSC Quality Assurance Officer Signature:	<u>Susan M. D. Conn</u>		<u>1/23/85</u>
Sponsor Representative Signature (if applicable):	<u>Charles S. Piper</u>		<u>1-28-85</u>
Telephone Authorization of Sponsor (if applicable):	<u>Dr. Charles Piper</u>		<u>1/18/85</u>

Bioassay Systems CorporationProtocol Amendment Form

Sponsor Name: GDS
BSC Project Number: 1257 BSC Sample Number: 871226
Study Title: Ames Test
Protocol Amendment Number: 7

Section 3.6

A second aliquot of the test substance
was received on 10/31/84.

	Date
Study Director Signature: <u>Lisa M. Perini</u>	<u>2/5/85</u>
BSC Quality Assurance Officer Signature: <u>Nancy Perini</u>	<u>2/5/85</u>
Sponsor Representative Signature (if applicable): <u>Charles E. Piper</u>	<u>2/7/85</u>
Telephone Authorization of Sponsor (if applicable): _____	

Bioassay Systems CorporationProtocol Amendment FormSponsor Name: G.D. Seale & CoBSC Project Number: 12157BSC Sample Number: 84-1226 A, BStudy Title: AmesProtocol Amendment Number: #8

Section 4.3.8 Stability: (solution) at least 24 hrs. at room temperature
 - changed to reflect current stability information.

Section 3.6 Changes made to section 3.6 in Amendment #'s 1, 2, 4, 6 were made to reflect actual initiation and completion dates of the study.

Section 4.3.10 Changes made to section 4.3.10 in Amendment #2 were made to reflect current procedures used in the preparation & use of 2-Anthracene

Section 6.3 Additions made to section 6.3 in Amendment #'s 2 & 4 were made to complete information specified in the protocol.

Section 6.4 Changes made to section 6.4 in Amendment #2 were made to clarify the procedures used to count (microplates), since both automatic & manual counting is performed.

Section 4.1.11 Changes made to section 4.1.11 in Amendment #3 were made to reflect current solubility information.

Study Director Signature: Veronika F. [Signature] Date: 3/18/85
 BSC Quality Assurance Officer Signature: Susan M. O. [Signature] 3/16/85
 Sponsor Representative Signature (if applicable): Charles E. [Signature] 3-21-85
 Telephone Authorization of Sponsor (if applicable): N/A

Bioassay Systems CorporationProtocol Amendment Form

Sponsor Name: G.D. Searle & Co
BSC Project Number: 12157 BSC Sample Number: 84-1226 A+B
Study Title: Ames
Protocol Amendment Number: #8 (con't)

Section 4.0 additions made to section 4.0 in Amendment # 5 were made to clarify the methodology used in the analysis of the test article in the carrier.

Section 3.6 additions to section 3.6 in Amendment # 7 were made to document the receipt of an additional test sample aliquot.

Study Director Signature: [Signature] Date: 3/18/85
BSC Quality Assurance Officer Signature: [Signature] 3/16/85
Sponsor Representative Signature (if applicable): Charles E. Piper 3-21-85
Telephone Authorization of Sponsor (if applicable): N/A

R&D PRODUCT DEVELOPMENT FUNCTION
REPORT REVIEW AND RELEASE

Page 1 of 6

DEPARTMENT: Product Development Analytical

DOCUMENT NUMBER: F-317-034-05

TITLE OF REPORT: SC-19129

TYPE OF REPORT: Analytical Summary in Support of Product Safety
Assessment Study Number 2462

AUTHOR(S): Charles Demarest

Charles Demarest 3-12-85

TECHNICAL WRITER: Michele Newcomb

Michele Newcomb 3/14/85

APPROVAL: James Jiu

James Jiu 14-march-85REVIEWED BY: ReviewerSignatureDate

Daniel Sweeney

Daniel Sweeney3-12-85

Kathy Klimovitz

Kathryn P. Klimovitz3-12-85

APPROVAL FOR RELEASE:

R. Baum
R. Baum, Director
Analytical Development3/19/85
DateLarry Hansen
L. Hansen,
Senior Director
Product Development3/19/85
DateNORTH AMERICAN PRECLINICAL RESEARCH AND DEVELOPMENT
SEARLE PHARMACEUTICALS AND CONSUMER PRODUCTS
SKOKIE, ILLINOIS

Subject: SC-19129

Summary Number: F-317-034-05

Applicable to SA Study Number: 2462

Test Article Characterization and Stability

Lot 84K-047-101 was analyzed using the release methods of testing, released against the current specifications (NS-S84-015-A), and given a re-evaluation period of one year prior to use in this study.

A summary of the significant results used to characterize the SC-19129 is presented in Table 1.

Table 1

	Pre-Study	Post-Study	
Analysis Report #	84N1058	85N0093	85N0094
Completion Date	10/16/84	02/15/85	02/15/85
Identity (HPLC)	Conforms to Standard	Conforms to Standard	Conforms to Standard
Assay (HPLC)	100.0% n = 3 s = 0.2	98.9% n = 3 s = 0.8	99.1% n = 3 s = 0.5
Water	9.8%	8.7%	8.4%

These results and all other results, coupled with the use of lot 84K-047-101 within its re-evaluation period, indicate that lot 84K-047-101 of SC-19129 was suitable for use in this study.

Subject: SC-19129

Summary Number: F-317-034-05

Applicable to SA Study Number: 2462

Stability of Test Article in Carrier

The stability of SC-19129 in 100 mg/mL solutions of dimethyl sulfoxide was determined using a stability indicating HPLC method (M84-046-A). The samples were stored at ambient conditions and sampled at times 0, 1, 2, 4, 8, 24, 48, and 72 hours. The results of the analysis are presented in Table 2. The statistics, using data at t = 0 as reference, are based on the percent relative recovery values.

The results of the linear regression analysis (MINITAB, Reference 1) gave a t value less than the table value (Reference 2, Table A-4), indicating no significant downward trend. The correlation between the observed recovery values and the predicted values exhibited a normal probability plot (References 1 and 3). Since the slope of the regression line showed no significant downward trend, and the differences between the observed recovery values and the predicted values were normally distributed, the SC-19129 in solutions of dimethyl sulfoxide at 100 mg/mL is considered stable for at least 72 hours at ambient conditions.

Notebook Reference: K. Klimovitz PDAD61 pp. 160-176

ANALYTICAL SUMMARY
Product Development Analytical Department

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Subject: SC-19129

Summary Number: F-317-034-05

Applicable to SA Study Number: 2462

Table 2

Stability of Test Article in Carrier

100 mg of SC-19129/mL of Dimethyl Sulfoxide

Report of Analysis # 84-2365

Time (Hours)	% SC-19129 Recovered	% Relative Recovery
0	100.8 101.2 100.8 99.7	Reference X = 100.6
1	100.4 102.3	99.8 101.7
2	103.9 100.8	103.3 100.2
4	101.6 100.4	101.0 99.8
8	101.5 100.5	100.9 99.9
24	100.5 100.2	99.9 99.6
48	99.1 99.4	98.5 98.8
72	101.0 100.6	100.4 100.0
Intercept		100.7
Slope		- 0.020
t-Ratio		- 1.69
t(0.95, 12 df)		1.789
Correlation Coefficient Predicted vs Observed		0.945

ANALYTICAL SUMMARY
Product Development Analytical Department

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Subject: SC-19129

Summary Number: F-317-034-05

Applicable to SA Study Number: 2462

References:

1. Ryan, Jr., T. A., Joiner, B. L., and Ryan, B. F., "MINITAB Student Handbook", 1976, Wadsworth Publishing Co., Inc.
2. Natrella, M. G., "Experimental Statistics, National Bureau of Standards Handbook 91", 1963, US Government Printing Office
3. Filliben, J., Technometrics, 17 (1), 111 (1975)

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Product Development Analytical Department

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Subject: SC-19129

Summary Number: F-317-034-05

Applicable to SA Study Number: 2462

GLP Compliance Statement

To the best of our knowledge, the support activities provided by the Product Development Analytical Department for this study were conducted in compliance with the Good Laboratory Practices Regulations, as set forth in part 58, 21 CFR.