

SC-19192: AN EVALUATION OF MUTAGENIC POTENTIAL
EMPLOYING THE HOST-MEDIATED ASSAY IN THE MOUSE

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INTRODUCTION

This study employed the host-mediated assay and was designed to measure the mutagenic potential of SC-19192. This assay procedure was originally described by Gabridge and Legator (Proc. Soc. Exp. Biol. Med., 30:821-834, 1969). The assay employs a bacterial indicator system, Salmonella typhimurium G-46, a histidine auxotroph, and attempts to test indirectly for mutagenic activity in mammalian systems. In this study, Sprague-Dawley Ha/ICR random bred Swiss male mice were used as the mammalian host. The animals were treated for five consecutive days and following the final dose on the fifth day the host-mediated assay was employed.

MATERIALS AND METHODS

SC-19192, diketopiperazine (DKP), was supplied by Ajinomoto Co. (Lot No. 6-R). The mice were Ha/ICR random bred Swiss males (5-6 weeks of age) which were obtained from Sprague-Dawley (Madison, Wisc.). The mice were housed individually in elevated wire mesh cages. They were fed Purina Rat Chow and were given water ad libitum. Body weights were measured immediately prior to the initiation of treatment and immediately after the final dose on the fifth day. Food consumption was measured after completion of treatment. The bacterial indicator system Salmonella typhimurium G-46, a histidine

auxotroph containing a point mutation, has been maintained in this laboratory on nutrient agar slants. All bacteriological reagents were obtained from Difco Laboratories (Detroit, Mich.). Methylazoxymethanol acetate, which was used as a positive control, was obtained from Schwarz/Mann Bioreserch Co. (Orangeburg, N.Y.).

Sixty male albino mice were selected randomly and divided into the following groups:

Group Number	Treatment	No. of Animals (sex)	Dosage (g/kg/day)	Multiple of Estimated Daily Human (27 kg) Intake**
1	Vehicle Control (1% aqueous Tween-80)	10 (males)	40 ml/kg/day	--
2	Positive Control (Methylazoxymethanol)	10 (males)	0.020*	--
3	SC-19192 (Low)	10 (males)	1	133
4	SC-19192 (Med)	10 (males)	2	266
5	SC-19192 (High)	10 (males)	4	532
6	SC-19192 (V. High)	10 (males)	8	1064

* Single dose administered IG as a 0.2% solution (v/v) in sterile isotonic NaCl.

**Based on 7.5 mg/kg daily intake. Assumes a 25% decomposition of a 30 mg/kg SC-18862 dose to a 27 kg child.

With the exception of the positive control group, all the animals were treated with three equally divided daily doses at two hour intervals for five consecutive days. At the low and medium dose levels of SC-19192, the compound was administered as a 10% suspension in 1% aqueous Tween-80. At the high and very high levels, SC-19192 was administered as a 15% suspension in 1% aqueous Tween-80. The positive control group received methylazoxymethanol acetate in a single dose 30 minutes prior to instituting the host-mediated assay. All compounds were administered via the intragastric route.

The large number of animals in the study precluded the possibility of treating all the animals and performing the host-mediated assay simultaneously. Therefore, treatment was initiated on two animals from each treatment group (twelve animals) on each of five consecutive days as illustrated in the following table:

Days	Animals				
	1 & 2	3 & 4	5 & 6	7 & 8	9 & 10
	<u>Treatment Day</u>				
1	Initial				
2	Second	Initial			
3	Third	Second	Initial		
4	Fourth	Third	Second	Initial	
5	Fifth	Fourth	Third	Second	Initial
6		Fifth	Fourth	Third	Second
7			Fifth	Fourth	Third
8				Fifth	Fourth
9					Fifth

Thus, the host-mediated assay was performed on twelve mice per day for five consecutive days.

Thirty minutes after the final dosing, all mice received, by intraperitoneal injection, 1.0 ml of an 18 hour nutrient broth culture of Salmonella typhimurium G-46, a histidine auxotroph. The culture was prepared by removing a small quantity of bacteria with an inoculating loop from a stock nutrient agar slant which was maintained at 4°C. The broth

culture was incubated at 37°C in a reciprocating shaker-incubator. Immediately before the bacteria were injected into the mammalian host, a 10^{-6} dilution of a small aliquot of the culture was made. One-tenth ml of this 10^{-6} dilution was plated on each of three nutrient agar plates to determine the total number of colony-forming units/ml in the overnight culture.

Three hours after the injection of bacteria the animals were sacrificed by CO₂ inhalation. The abdominal region was then swabbed with 95% ethanol and 2.0 ml of sterile saline was injected into the peritoneal cavity. The cavity was opened under aseptic conditions and as much of the peritoneal exudate as possible was expelled into a chilled, sterile Wassermann tube and removed by a 3 ml syringe (without needle). The exudate was maintained at 4°C until plated. Dilution blanks were prepared in advance and were also chilled. Hundred-fold serial dilutions were made of each peritoneal exudate yielding a concentration from 10⁰ (undiluted) through 10⁻⁶.

To determine the total bacterial count in the peritoneal exudate of each animal, 0.1 ml of the 10^{-6} dilution was plated on each of three nutrient agar plates. For determining the total mutant colony count, Spizizen's minimal agar, a minimal medium without histidine, served as the selective medium. Two-tenths ml of the undiluted peritoneal extract was plated on a minimal agar plate (4 plates/sample). To spread the bacteria over the surface of both the nutrient agar plates and Spizizen's plates, each plate was placed on a petri dish turntable. The table was spun and the sample was spread over the surface by a bent glass rod which had been immersed in 95% ethanol and flamed before use. All plates were incubated at 37°C. The nutrient agar plates were incubated for 18 hours and the minimal agar plates for 40 hours.

A determination of the mutation frequency was made in the following manner:

$$\frac{\text{No. of colonies/sample}}{\text{No. of plates}} \times \text{plating factor} = \text{CFU*/ml of sample plated}$$

$$\text{CFU/ml} \times \text{dilution factor} = \text{CFU/ml in undiluted exudate}$$

$$\text{MF}^\dagger = \frac{\text{CFU/ml (mutant colonies)}}{\text{CFU/ml (total colonies)}}$$

*CFU - colony-forming units

† MF - mutation frequency

Analysis of variance was employed to compare the control with each treated group mean in the evaluation of data for body weights, mean mutation frequencies, the mean number of total colonies and the mean number of mutant colonies.

RESULTS

Survival after the five day treatment phase was 100%, 100%, 100%, 90%, 80%, and 30% for the vehicle control, positive control, low, medium, high and very high doses, respectively. One animal from the medium dose group (2 g/kg/day) died following the third dose on the third day. Autopsy revealed the presence of compound in the lungs. Two animals from the high dose group (4 g/kg/day) died following the third dose on the third day. Autopsy of one animal revealed the presence of a large blood clot in the pericardium; cause of the clot was not determined. Contributing to the apparent cause of death of the second animal were severe diarrhea and subsequent dehydration; autopsy revealed the presence of a liquified fecal material in the cecum. In the very high dose group (8 g/kg/day), seven of ten animals died during the treatment period. Deaths occurred as early as the third dose on the second day and as late as the first dose on the fifth day. All of these animals exhibited signs of severe diarrhea and subsequent dehydration. All animals in the high and very high dose groups which survived the treatment period showed signs of marked diarrhea.

Several animals which survived the treatment phase of the study died following intraperitoneal injection of the bacterial indicator system. Of the animals that died (one vehicle control, two low dose and one high dose animal), three of the four exhibited a marked weight loss (Appendix Table 1).

All of the treated and vehicle control groups exhibited a loss in mean body weight (Table 1). However, the terminal weights of those animals which received SC-19192 did not differ significantly from the

Table 1

SC-19192: AN EVALUATION OF MUTAGENIC POTENTIAL
EMPLOYING THE HOST-MEDIATED ASSAY IN THE MOUSE

Mean Body Weight and Food Consumption Data (\pm S.E.)

Treatment Group and Dose	N	Mean Body Weight (g)		Mean Food Consumption (g)	
		Day 0	Day 5	Five Days	
Vehicle Control 1% Aqueous Tween-80 40 ml/kg/day	10	24.4 \pm 0.5	21.5 \pm 0.8	19.6 \pm 1.1	
Positive Control Methylazoxymethanol 20 mg/kg	10	24.6 \pm 0.6	25.3 \pm 1.6	24.7 \pm 1.1*	
Low Dose SC-19192 1 g/kg/day	10	24.0 \pm 0.6	21.7 \pm 1.2	17.8 \pm 1.9	
Medium Dose SC-19192 2 g/kg/day	9	25.2 \pm 0.6	22.6 \pm 1.1	20.1 \pm 1.8	
High Dose SC-19192 4 g/kg/day	8	24.3 \pm 0.7	21.8 \pm 0.4	18.5 \pm 0.9	
Very High Dose SC-19192 8 g/kg/day	3	23.0 \pm 0.6	21.3 \pm 0.7	16.7 \pm 0.9	

* Mean differs significantly from control ($p < 0.05$).

vehicle control. Also the food consumption of those animals treated with SC-19192 was not significantly different from the vehicle control. The positive control group, which was not handled during the treatment period, did not experience a weight loss and food consumption was also significantly higher than the treated groups.

A valid host-mediated assay is predicated on adequate replication of the bacteria within the peritoneal cavity of the mammalian host. From the peritoneal exudate the total number of bacterial cells is determined. If no significant difference exists between control and treated groups or if the total counts in the treated groups are higher than the control group, the assumption can be made that bacterial replication is not inhibited by the test material. A second indication of adequate replication is found in the positive control group. If an increased mutation frequency is manifested by this group, this suggests that bacterial replication has occurred within the host.

As indicated in Table 2, the mean total colony count/ml of the treated groups was greater than the count obtained from the vehicle control group. Also, the mutation frequency of the positive control group was significantly higher than the vehicle control (Table 3). These results suggest that the bacteria replicated adequately to produce a valid assay.

Results of the host-mediated assay indicated that the mean mutation frequencies (Table 3) were not increased in the SC-19192 treated groups as compared to the vehicle control group. In fact, in the low and very high dose groups the mutation frequency is significantly lower than in the vehicle control. Therefore, at the dose levels reported herein, results of the host-mediated assay indicate that SC-19192 does not possess measurable mutagenic activity.

Table 2

SC-19192: AN EVALUATION OF MUTAGENIC POTENTIAL
EMPLOYING THE HOST-MEDIATED ASSAY IN THE MOUSE

Mean Total Colony Counts and Mean Mutant Colony Counts (\pm S.E.)

Treatment Group and Dose	N	Mean Total Colony Count/ml \pm S.E. ($\times 10^8$)	Mean Mutant Colony Count/ml \pm S.E.
Vehicle Control 1% Aqueous Tween-80 40 mg/kg/day	6	13.8 \pm 2.0	6.5 \pm 1.3
Positive Control Methylazoxymethanol 20 mg/kg	7	18.0 \pm 3.0	34.6 \pm 5.3**
Low Dose SC-19192 1 g/kg/day	7	22.8 \pm 2.9*	4.5 \pm 1.0
Medium Dose SC-19192 2 g/kg/day	9	17.0 \pm 1.6	8.1 \pm 0.9
High Dose SC-19192 4 g/kg/day	7	19.2 \pm 3.6	6.8 \pm 1.2
Very High Dose SC-19192 8 g/kg/day	3	26.6 \pm 2.3*	3.3 \pm 0.8

* Mean differs significantly from control ($p < 0.05$).

** Mean differs highly significantly from control ($p < 0.01$).

Table 3

SC-19192: AN EVALUATION OF MUTAGENIC POTENTIAL
EMPLOYING THE HOST-MEDIATED ASSAY IN THE MOUSE

Mean Mutation Indices

Treatment Group and Dose	No. of Mice	Mean Mutation Frequency (\pm S.E.)	Mft/MFc†
Vehicle Control 1% Aqueous Tween-80 40 ml/kg/day	6	$0.502 \times 10^{-8} \pm 0.109$	1.000
Positive Control Methylazoxymethanol 20 mg/kg	7	$2.153 \times 10^{-8} \pm 0.319^{**}$	4.289
Low Dose SC-19192 1 g/kg/day	7	$0.227 \times 10^{-8} \pm 0.048^{*}$	0.452
Medium Dose SC-19192 2 g/kg/day	9	$0.504 \times 10^{-8} \pm 0.077$	1.003
High Dose SC-19192 4 g/kg/day	7	$0.454 \times 10^{-8} \pm 0.110$	0.904
Very High Dose SC-19192 8 g/kg/day	3	$0.130 \times 10^{-8} \pm 0.044^{*}$	0.258

† Mft/MFc = $\frac{\text{mutation frequency of the experimental group}}{\text{mutation frequency of the control group}}$

* Mean differs significantly from control ($p < 0.05$).

** Mean differs highly significantly from control ($p < 0.01$).

SUMMARY AND CONCLUSIONS

The mutagenic potential of SC-19192 has been investigated by using the host-mediated assay. The assay utilizes a microbial indicator system and attempts to test for mutagenic activity in a mammalian host. In this study male Sprague-Dawley Ha/ICR random bred Swiss mice served as the mammalian host. Salmonella typhimurium G-46, a strain of Salmonella lacking the ability to synthesize histidine, served as the bacterial indicator system and was administered IP 30 minutes after the final dosing. The mice were treated with SC-19192 in dosages of 1, 2, 4 and 8 g/kg/day, administered orally in three daily divided doses 2 hours apart for five consecutive days. A vehicle control group composed of mice treated with 1% aqueous solution of Tween-80 (40 ml/kg/day) and a positive control group consisting of mice treated with a single dose of methylazoxymethanol acetate (20 mg/kg) 30 minutes prior to instituting the host-mediated assay were also employed.

Diarrhea and resulting dehydration occurred at higher dosage levels of SC-19192, and appeared responsible for the high mortality rate (70%) observed at the 8 g/kg/day level.

Results indicate that SC-19192, administered intragastrically in doses up to 8 g/kg/day for a period of five consecutive days, does not exhibit a mutagenic effect in the host-mediated assay in the mouse.

Tables of Individual Values

APPENDIX

Appendix Table 1

SC-19192: AN EVALUATION OF MUTAGENIC POTENTIAL
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Individual Body Weights

Group No.	Animal No.	Weight (g)	
		Pretreatment	Post-Treatment
1 Vehicle Control 1% Aqueous Tween-80 40 ml/kg/day	1	23	21
	2	25	22
	3	†† 25	17
	4	26	24
	5	24	19
	6	23	20
	7	24	25
	8	22	23
	9	27	23
	10	25	21

†† Died post-bacterial injection.

Appendix Table 1 (cont.)

SC-19192: AN EVALUATION OF MUTAGENIC POTENTIAL
EMPLOYING THE HOST-MEDIATED ASSAY IN THE MOUSE

Individual Body Weights

Group No.	Animal No.	Weight (g)	
		Pretreatment	Post-Treatment
2 Positive Control Methylazoxymethanol Acetate 20 mg/kg	1	25	23
	2	25	23
	3	26	25
	4	21	28
	5	25	24
	6	26	27
	7	22	23
	8	23	27
	9	26	27
	10	27	26

Appendix Table 1 (cont.)

SC-19192: AN EVALUATION OF MUTAGENIC POTENTIAL
EMPLOYING THE HOST-MEDIATED ASSAY IN THE MOUSEIndividual Body Weights

Group No.	Animal No.	Weight (g)	
		Pretreatment	Post-Treatment
3 Low Dose SC-19192 1 g/kg/day	1	24	22
	2	†† 22	15
	3	24	21
	4	22	18
	5	25	25
	6	27	26
	7	24	25
	8	23	22
	9	27	25
	10	†† 22	18

†† Died post-bacterial injection.

Appendix Table 1 (cont.)

SC-19192: AN EVALUATION OF MUTAGENIC POTENTIAL
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Individual Body Weights

Group No.	Animal No.	Weight (g)	
		Pretreatment	Post-Treatment
4 Medium Dose SC-19192 2 g/kg/day	1	23	21
	2	23	19
	3	26	25
	4	25	23
	5	25	16
	6	27	26
	7	Died Before Assay	
	8	22	24
	9	27	25
	10	27	24

Appendix Table 1 (cont.)

SC-19192: AN EVALUATION OF MUTAGENIC POTENTIAL
EMPLOYING THE HOST-MEDIATED ASSAY IN THE MOUSE

Individual Body Weights

Group No.	Animal No.	Weight (g)	
		Pretreatment	Post-Treatment
5 High Dose SC-19192 4 g/kg/day	1	25	21
	2	23	21
	3	++ 26	22
	4	25	22
	5	Died Before Assay	
	6	28	23
	7	23	23
	8	22	22
	9	23	20
	10	Died Before Assay	

++ Died post-bacterial injection.

Appendix Table 1 (cont.)

SC-19192: AN EVALUATION OF MUTAGENIC POTENTIAL
EMPLOYING THE HOST-MEDIATED ASSAY IN THE MOUSE

Individual Body Weights

Group No.	Animal No.	Weight (g)	
		Pretreatment	Post-Treatment
6 Very High Dose SC-19192 8 g/kg/day	1		Died Before Assay
	2		Died Before Assay
	3		Died Before Assay
	4		Died Before Assay
	5	24	22
	6		Died Before Assay
	7	23	20
	8	22	22
	9		Died Before Assay
	10		Died Before Assay

Appendix Table 2

SC-19192: AN EVALUATION OF MUTAGENIC POTENTIAL
EMPLOYING THE HOST-MEDIATED ASSAY IN THE MOUSE

Cell Counts and Mutation Frequencies

Group No.	Mouse No.	Total Cells CFU/ml of Exudate	Mutant Cells CFU/ml of Exudate	Mutation Frequency
1 Vehicle Control 1% Aqueous Tween-80 40 ml/kg/day	1	15.5×10^8	5.00	0.323×10^{-8}
	2	23.8×10^8	7.50	0.315×10^{-8}
	3	Died Post-Bacterial Injection		
	4	11.17×10^8	10.00	0.895×10^{-8}
	5	5.8×10^8	Contamination	
	6	17.63×10^8	10.00	0.567×10^{-8}
	7	19.70×10^8	Contamination	
	8	13.17×10^8	Contamination	
	9	5.30×10^8	3.75	0.707×10^{-8}
	10	12.37×10^8	2.50	0.202×10^{-8}
	Average	13.83×10^8		

Appendix Table 2 (cont.)

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EMPLOYING THE HOST-MEDIATED ASSAY IN THE MOUSE

Cell Counts and Mutation Frequencies

Group No.	Mouse No.	Total Cells CFU/ml of Exudate	Mutant Cells CFU/ml of Exudate	Mutation Frequency
2 Positive Control Methylazoxymethanol Acetate 20 mg/kg	1	18.60×10^8	Contamination	
	2	16.40×10^8	38.75	2.36×10^{-8}
	3	21.13×10^8	Contamination	
	4	40.30×10^8	50.00	1.24×10^{-8}
	5	15.30×10^8	36.25	2.37×10^{-8}
	6	25.10×10^8	52.50	2.09×10^{-8}
	7	12.53×10^8	20.00	1.60×10^{-8}
	8	12.23×10^8	Contamination	
	9	7.87×10^8	30.00	3.81×10^{-8}
	10	10.20×10^8	15.00	1.60×10^{-8}
	Average	17.96×10^8		

Appendix Table 2 (cont.)

SC-19192: AN EVALUATION OF MUTAGENIC POTENTIAL
EMPLOYING THE HOST-MEDIATED ASSAY IN THE MOUSECell Counts and Mutation Frequencies

Group No.	Mouse No.	Total Cells CFU/ml of Exudate	Mutant Cells CFU/ml of Exudate	Mutation Frequency
3 Low Dose SC-19192 1 g/kg/day	1	18.70 x 10 ⁸	3.75	0.201 x 10 ⁻⁸
	2	Died Post-Bacterial Injection		
	3	29.97 x 10 ⁸	1.25	0.042 x 10 ⁻⁸
	4	31.73 x 10 ⁸	7.50	0.236 x 10 ⁻⁸
	5	33.20 x 10 ⁸	Contamination	
	6	20.80 x 10 ⁸	8.75	0.421 x 10 ⁻⁸
	7	17.67 x 10 ⁸	2.50	0.141 x 10 ⁻⁸
	8	19.70 x 10 ⁸	3.75	0.190 x 10 ⁻⁸
	9	10.53 x 10 ⁸	3.75	0.356 x 10 ⁻⁸
	10	Died Post-Bacterial Injection		
Average		22.79 x 10 ⁸		

Appendix Table 2 (cont.)

SC-19192: AN EVALUATION OF MUTAGENIC POTENTIAL
EMPLOYING THE HOST-MEDIATED ASSAY IN THE MOUSECell Counts and Mutation Frequencies

Group No.	Mouse No.	Total Cells CFU/ml of Exudate	Mutant Cells CFU/ml of Exudate	Mutation Frequency
4 Medium Dose SC-19192 2 g/kg/day	1	15.2×10^8	8.75	0.568×10^{-8}
	2	22.17×10^8	11.25	0.507×10^{-8}
	3	18.97×10^8	8.75	0.461×10^{-8}
	4	10.07×10^8	10.00	0.993×10^{-8}
	5	16.13×10^8	8.75	0.542×10^{-8}
	6	25.63×10^8	8.75	0.341×10^{-8}
	7	Died During Treatment Period		
	8	13.67×10^8	8.75	0.640×10^{-8}
	9	14.77×10^8	3.75	0.254×10^{-8}
	10	16.00×10^8	3.75	0.234×10^{-8}
	Average	16.98×10^8		

Appendix Table 2 (cont.)

SC-19192: AN EVALUATION OF MUTAGENIC POTENTIAL
EMPLOYING THE HOST-MEDIATED ASSAY IN THE MOUSECell Counts and Mutation Frequencies

Group No.	Mouse No.	Total Cells CFU/ml of Exudate	Mutant Cells CFU/ml of Exudate	Mutation Frequency
5 High Dose SC-19192 4 g/kg/day	1	29.70×10^8	5.00	0.169×10^{-8}
	2	16.03×10^8	12.50	0.780×10^{-8}
	3	Died Post-Bacterial Injection		
	4	21.17×10^8	10.00	0.472×10^{-8}
	5	Died During Treatment Period		
	6	31.00×10^8	5.00	0.161×10^{-8}
	7	5.53×10^8	5.00	0.904×10^{-8}
	8	9.13×10^8	3.75	0.411×10^{-8}
	9	21.97×10^8	6.25	0.284×10^{-8}
	10	Died During Treatment Period		
	Average	19.2×10^8		

Appendix Table 2 (cont.)

SC-19192: AN EVALUATION OF MUTAGENIC POTENTIAL
EMPLOYING THE HOST-MEDIATED ASSAY IN THE MOUSECell Counts and Mutation Frequencies

Group No.	Mouse No.	Total Cells CFU/ml of Exudate	Mutant Cells CFU/ml of Exudate	Mutation Frequency
6 Very High Dose SC-19192 8 g/kg/day	1	Died During Treatment Period		
	2	Died During Treatment Period		
	3	Died During Treatment Period		
	4	Died During Treatment Period		
	5	23.03×10^8	5.00	0.217×10^{-8}
	6	Died During Treatment Period		
	7	26.03×10^8	2.50	0.092×10^{-8}
	8	30.77×10^8	2.50	0.081×10^{-8}
	9	Died During Treatment Period		
	10	Died During Treatment Period		
Average		26.61×10^8		