

Report on Mutagenicity Assay of Boswellin CG

Purpose:

The purpose of this study is to evaluate the mutagenic potential of the test article by measuring its ability to induce reverse mutations at selected loci of *Salmonella typhimurium* TA102.

Test & Control Substances:

Test Article: Boswellin CG

Controls : Negative: Test article vehicle
Positive: Methyl methanesulfonate.

Test System:

The tester strain is *S. typhimurium* histidine auxotrophs TA102

Genotype of the tester strain used for Mutagen testing are

Histidine mutation

Additional mutations : LPS (rfa), Repair(uvrB),R-factor (Ampicillin resistance).

S. typhimurium TA102 tester strain contains, in addition to a mutation in the histidine operon, additional mutations that enhance sensitivity to some mutagens. The rfa mutation results in a cell wall deficiency that increases the permeability of the cell to certain classes of chemicals such as those containing large ring systems that would otherwise be excluded. The deletion in the uvrB gene results in a deficient DNA excision repair system. And R-factor, PAQ1 plasmid which carries the hisG428 mutation and Tetracycline resistance gene.

The *S. typhimurium* TA102 strain was received from Bonnie Kessing, Discovery partners, Xenometrix, Sandoz CA Fax(858)455-8085

Experimental Design and Methodology

The test article will be tested at a minimum of five dose levels along with appropriate negative and positive control with tester strain TA102. All dose levels of test article, negative controls & positive controls will be plated in triplicate.

Solubility:

Solubility determination will be conducted to determine the maximum soluble concentration. Vehicles compatible with this test system in order of preference include deionized water, DMSO, Ethanol, and Acetone. The vehicle of choice will be the solvent,

in order of preference, which permits preparation of the highest workable/soluble stock concentration

Preliminary Toxicity Assay to select Dose levels

Selection of dose levels for the mutagenicity assay will be based upon the toxicity and precipitation profile of the test article. The preliminary assay will be conducted by exposing TA 102 to negative controls and to at least 8 concentrations of test article, one plate per dose level. The highest workable concentration in the vehicle of choice but not to exceed 5 mg/plate. Doses will be selected such that precipitate does not interfere with manual scoring. Whenever possible, the highest dose for the mutagenicity assay will be selected to give some indication of toxicity without exceeding 5mg/plate.

Dose level is considered toxic if it causes a >50% reduction in the mean number of revertants per plate relative to the mean negative control value or a reduction in the background lawn.

Code	Description	Characteristics
1	Normal	Distinguished by a healthy microcolony lawn
2	Slightly reduced	Distinguished by a noticeable thinning of the microcolony lawn possibly a slight increase in the size of the microcolonies compared to the vehicle control plate.
3	Moderately reduced	Distinguished by a marked thinning of the microcolony lawn resulting in a pronounced increase in the size of the microcolonies compared to the vehicle control plate.
4	Extremely reduced	Distinguished by an extreme thinning of the microcolony lawn resulting in an increase in the size of the microcolonies compared to the vehicle control plate such that the microcolony lawn is visible to the unaided eye as isolated colonies.
5	Absent	Distinguished by a complete lack of any microcolony lawn over >90% of the plate.
6	Obscured by Precipitate	The background bacterial lawn cannot be accurately evaluated due to microscopic test article precipitate.
NP	Non-Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye but any precipitate particles detected by the automated colony counter total less than 10% of the revertant colony count (e.g., < 3 particles on a plate with 30 revertants.)
IP	Interfering Precipitate	Distinguished by precipitate on the plate that is visible to the naked eye and any precipitate particles detected by the automated colony counter exceed 10% of the revertant colony count (e.g., >3 particles on a plate with 30 revertants.)

Preparation of Tester strain

S. typhimurium TA102(40µl) will be inoculated into 5ml of nutrient broth from the appropriate frozen stock at the end of the day. Inoculated tubes are placed in a shaker/incubator and run at 125 rpm & at 37°C for 12 hrs.

Test system identification

Each plate will be labeled with a code system that identifies the test article, dose level, tester strain.

Test article preparation

Test article dilutions will be prepared immediately prior to use. All test dosing will be at room temperature.

Treatment of test system

100µl of tester strain and 50µl of vehicle, test article dilution or positive control will be added to 2.0ml of molten top agar at 45±2°C. The mixture will be vortex mixed and overlaid onto the surface of 25ml of minimal bottom agar. After the over layer has solidified, the plates will be inverted and incubated for 48 –72 hrs. at 37±2°C. Plates that are not counted immediately following the incubation period will be stored at 2-8°C.

Scoring

The condition of the bacterial background lawn will be evaluated for evidence of test article toxicity and precipitate. Toxicity will be evaluated as a decrease in the number of revertant colonies per plate and/or a thinning or disappearance of the bacterial background lawn. Precipitation will be evaluated after the incubation period by visual examination without magnification.

Tester strain verification

In addition to His operon, the tester strain should be checked for the appropriate genetic markers. To demonstrate the presence of the *rfa* mutation, all *S. typhimurium* tester strain cultures must exhibit sensitivity to crystal violet. To demonstrate the presence of the *uvrB* mutation, all *S. typhimurium* tester strain cultures must exhibit sensitivity to UV-light. To demonstrate the presence of R-factor plasmid & PAQ1 plasmid, all *S. typhimurium* tester strain cultures must exhibit resistance to Ampicillin & Tetracycline.

Tester strain Titers

To ensure that appropriate numbers of bacteria are plated, tester strain culture titer must be equal to or greater than 0.3×10^9 cells per milliliter.

Positive Control Values

Each mean positive control values must exhibit at least a three-fold increase over the respective mean negative control value (vehicle).

Evaluation of Test results

For a test article to be evaluated positive, data sets must cause a dose- related increase (two-three folds) in the mean revertants per plate over a minimum of two increasing concentrations of test article. Data sets for tester strains TA102 were judged positive if the increase in the mean revertants at the peak of the dose response is equal to or greater than three times the mean vehicle control value

Table-I
Preliminary Toxicity Assay

Test Article Id : Boswellin CG
 Strain : *Salmonella typhimurium* TA102
 Date plated : 27.08.03
 Plating aliquot: 50µl
 Vehicle : Dimethyl sulphoxide

Test Article Concentration µg/plate	Revertants/plate	Background Lawn
Vehicle	60	1
207.0	43	1
415.0	42	1
830.0	36	1
1000.0	55	1
1250.0	49	1
1600.0	33	1
2500.0	38	NP
5000.0	43	NP

Background Lawn Code

1= Normal: 2= Slightly reduced: 3= Severely reduced: 4= Extremely reduced:
 5= Absent: 6= Obscured by precipitate: NP=Non-Interfering precipitate: IP:
 Interfering precipitate.

Table-II
Direct Bacterial Mutation Assay

Test article ID : Boswellin CG
 Batch No. : C20286/HI
 Strain : *Salmonella typhimurium* TA 102
 Vehicle : Dimethyl Sulphoxide Date plated : 27/08/2003
 Plating aliquot : 50µl Cells seeded: 0.5X10⁹

Concentration Mg/plate	Plate no.	Revertants per plate	Background code	Average revertants.
Vehicle	01	64	1	60
	02	60		
	03	58		
1.0	01	60	1	55
	02	54		
	03	52		
1.25	01	53	1	49
	02	50		
	03	45		
1.6	01	30	1	33
	02	34		
	03	36		
2.5	01	32	NP	38
	02	42		
	03	40		
5.0	01	46	NP	43
	02	40		
	03	43		
PositiveControl MethylMethaneSulfonate 1.5µl/plate	01	420	1	406
	02	410		
	03	390		

* note: background lawn code: 1- normal; 2- slightly reduced; 3- severely reduced; 4- extremely reduced; 5- absent; 6- obscured by precipitate; NP- non interfering precipitate; IP- interfering precipitate.

Results and Discussion

Solubility

Dimethyl Sulfoxide(DMSO) was selected as the solvent of choice based on compatibility with the target cells, and solubility of the test article. Concentrations from 75 to 100mg/ml were workable suspensions

Preliminary Toxicity Assay

The results of the preliminary toxicity assay are presented in table-1. In the toxicity assay, the maximum dose tested was 5000µg/plate: this dose was achieved using a concentration of 100mg/ml and a 50µl plating aliquot. Concentrations from 0.207mg to 5.0mg/plate were workable solutions.

Mutagenicity assay

The results of the mutagenicity assay are presented in Table-II. 1000µg to 5000µg/plate were taken for mutagenicity testing.

Conclusion

All criteria for a valid study were met as described in the protocol. The results of the Bacterial Reverse Mutation Assay indicate that, under the conditions of this study, Boswellin gave a negative response in the absence of Aroclor-induced rat liver S9.

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