FILE
Project No. B10-77-328

MUTAGENICITY EVALUATION

OF

BIO-77-328 CP 2321 SR

FINAL REPORT

SUBMITTED TO

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SPONSOR: Monsanto

MATERIAL: BIO-77-328 CP 2321 SR

SUBJECT: FINAL REPORT MUTAGENICITY PLATE ASSAY

1. OBJECTIVE

The objective of this study was to evaluate the test compound for genetic activity in microbial assays with and without the addition of mammalian metabolic activation preparations.

2. MATERIALS

A. Test Compound

1. Date Received: October 17, 1977

2. Description: Beige flakes

B. Indicator Microorganisms

Salmonella typhimurium, strains: TA-1535 TA-98
TA-1537 TA-100
TA-1538

Saccharomyces cerevisiae, strain: D4

C. Activation System (Ames et al., Mutation Research 31:347, 1975)

1. Reaction Mixture

Component	Final Concentration/ml
TPN	4 µmoles
Glucose-6-phosphate	5 µmoles
Sodium phosphate (dibasic)	100 µmoles
$MgCl_2$	8 µmoles
KCl	33 µmoles
Homogenate fraction equivalent to 25 mg of wet tissue	0.1-0.15 ml 9,000 x g supernatant of rat liver

2. S-9 Homogenate

A 9,000 x g supernatant was prepared from Sprague-Dawley adult male rat liver induced by Aroclor 1254 five days prior to kill.

2. MATERIALS (Continued)

D. Positive Control Chemicals

Table 1 below lists the chemicals used for positive controls in the nonactivation and activation assays.

TABLE 1

ASSAY	CHEMICAL[a]	SOLVENT	PROBABLE MUTAGENIC SPECIFICITY
Nonactivation	Methylnitrosoguanidine (MNNG)	Water or Saline	BPS[b]
	2-Nitrofluorene (NF)	Dimethylsulfoxide[c]	FS[b]
	Quinacrine mustard (QM)	Water or saline	FS[b]
Activation	2-Anthramine (ANTH)	Dimethylsulfoxide[c]	BPS[b]
	2-Acetylaminofluorene (AAF)	Dimethylsulfoxide[c]	FS[b]
	8-Aminoquinoline (AMQ)	Dimethylsulfoxide[c]	FS[b]

[a] Concentrations given in Results Section

[b] BPS = Base-pair substitution

FS = Frameshift

[c] Previously shown to be nonmutagenic

E. Solvent

Either deionized water or dimethylsulfoxide (DMSO) was used to prepare stock solutions of solid materials. All dilutions of test materials were made in either deionized water or DMSO. The solvent employed and its concentration are recorded in the Results Section.

3. EXPERIMENTAL DESIGN

A. Plate Test (Overlay Method*)

Approximately 10^8 cells from an overnight culture of each indicator strain were added to separate test tubes containing 2.0 ml of molten agar supplemented with biotin and a trace of histidine. For non-activation tests, at least four dose levels of the test compound were added to the contents of the appropriate tubes and poured over the surfaces of selective agar plates. In activation tests, a minimum of four different concentrations of the test chemical were added to the appropriate tubes with cells. Just prior to pouring, an aliquot of reaction mixture (0.5 ml containing the 9,000 x g liver homogenate) was added to each of the activation overlay tubes, which were then mixed, and the contents poured over the surface of a minimal agar plate and allowed to solidify. The plates were incubated for 48 hours at 37C, and scored for the number of colonies growing on each plate. The concentrations of all chemicals are given in the Results Section. Positive and solvent controls using both directly active positive chemicals and those that require metabolic activation were run with each assay.

B. Recording and Presenting Data

The numbers of colonies on each plate were counted and recorded on printed forms. These raw data were analyzed in a computer program and reported on a printout. The results are presented as revertants per plate for each indicator strain employed in the assay. The positive and the solvent controls are provided as reference points. Other relevant data are provided on the computer printout.

*Certain classes of chemicals known to be mutagens and carcinogens do not produce detectable responses using the standard Ames overlay method. Some dialkyl nitrosamines and certain substituted hydrazines are mutagenic in suspension assays, but not in the plate assay. Chemicals of these classes should be screened in a suspension assay.

IV RESULTS: TABLE I

A. NAME OR CODE DESIGNATION OF THE TEST COMPOUND: 810-77-328-CP-232158
B. SOLVENT: DMSO
C. TEST INITIATION DATE: OCT. 28, 1977
NOTE: CONCENTRATIONS ARE GIVEN IN MICROLITERS (UL) OR MICROGRAMS (UG) PER PLATE.

TEST	SPECIES	TISSUE	REVERTANTS PER PLATE											
			TA-1535		TA-1537		TA-1538		TA-98		TA-100		84*	
			1	2	1	2	1	2	1	2	1	2	1	2
NONACTIVATION														
SOLVENT CONTROL	---	---	11		11		21		35		212		69	
POSITIVE CONTROL **	---	---	938		729		631		895		956		>1000	
TEST COMPOUND														
0.100000 UG	---	---	6		9		9		28		220		111	
1.000000 UG	---	---	14		18		9		27		156		76	
10.000000 UG	---	---	14		15		5		32		171		102	
100.000000 UG	---	---	9		11		22		42		189		94	
500.000000 UG	---	---	9		11		12		50		176		99	
ACTIVATION														
SOLVENT CONTROL	RAT	LIVER	15		13		21		59		224		131	
POSITIVE CONTROL ***	RAT	LIVER	204		220		537		640		543		145	
TEST COMPOUND														
0.100000 UG	RAT	LIVER	10		2		24		20		192		90	
1.000000 UG	RAT	LIVER	19		8		15		40		222		82	
10.000000 UG	RAT	LIVER	15		8		12		60		218		95	
100.000000 UG	RAT	LIVER	15		1		13		37		242		99	
500.000000 UG	RAT	LIVER	27		9		22		46		230		100	

* TRY+ REVERTANTS PER PLATE

** TA-1535 NQNA 10 UG/PLATE
TA-1537 9A 10 UG/PLATE
TA-1538 NF 100 UG/PLATE
TA-98 NF 100 UG/PLATE
TA-100 NQNA 10 UG/PLATE
84 NQNA 10 UG/PLATE
SOLVENT DMSO 50 UL/PLATE

*** TA-1535 AMTH 100 UG/PLATE
TA-1537 AMO 100 UG/PLATE
TA-1538 AAF 100 UG/PLATE
TA-98 AAF 100 UG/PLATE
TA-100 AMTH 100 UG/PLATE
84 BNNA 100 MICROMOLES/PLATE
SOLVENT DMSO 50 UL/PLATE

MRNS 017967

5. INTERPRETATION OF RESULTS AND CONCLUSIONS

The test compound was examined for mutagenic activity in a series of in vitro microbial assays employing Salmonella and Saccharomyces indicator organisms. The compound was tested directly and in the presence of liver microsomal enzyme preparations from Aroclor-induced rats. The following results were obtained:

A. Toxicity Test Results

The compound was tested over a series of concentrations such that there was either quantitative or qualitative evidence of some chemically-induced physiological effects at the high dose level. The low dose in all cases was below a concentration that demonstrated any toxic effect. The dose range employed for the evaluation of this compound was from 0.1 µg to 500 µg per plate.

B. Nonactivation Test Results

The results of the tests conducted on the compound in the absence of a metabolic system were all negative.

C. Activation Test Results

The results of the tests conducted on the compound in the presence of the rat liver activation system were all negative.

D. Conclusions

The test compound, BIO-77-328 CP 2321 SR, did not demonstrate mutagenic activity in any of the assays conducted in this evaluation and was considered as not mutagenic under these test conditions.

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6. EXPLANATION OF EVALUATION PROCEDURES FOR PLATE ASSAYS

Plate test data consist of direct revertant colony counts obtained from a set of selective agar plates seeded with populations of mutant cells suspended in a semisolid overlay. Because the test chemical and the cells are incubated in the overlay for 2 to 3 days, and a few cell divisions occur during the incubation period, the test is semi-quantitative in nature. Although these features of the assay reduce the quantitation of results, they provide certain advantages not contained in a quantitative suspension test:

- The small number of cell divisions permits potential mutagens to act on replicating DNA, which is often more sensitive than nonreplicating DNA.
- The combined incubation of the compound and the cells in the overlay permits constant exposure of the indicator cells for 2 to 3 days.

A. Surviving Populations

Plate test procedures do not permit exact quantitation of the number of cells surviving chemical treatment. At low concentrations of the test chemical, the surviving population on the treatment plates is essentially the same as that on the negative control plate. At high concentrations, the surviving population is usually reduced by some fraction. Our protocol normally employs several doses ranging over two or three log concentrations, the highest of these doses being selected to show slight toxicity as determined by subjective criteria.

B. Dose Response Phenomena

The demonstration of dose-related increases in mutant counts is an important criterion in establishing mutagenicity. A factor that might modify dose-response results for a mutagen would be the selection of doses that are too low (usually mutagenicity and toxicity are related). If the highest dose is far lower than a toxic concentration, no increases may be observed over the dose range selected. Conversely, if the lowest dose employed is highly cytotoxic, the test chemical may kill any mutants that are induced, and the compound will not appear to be mutagenic.

6. EXPLANATION OF EVALUATION PROCEDURES FOR PLATE ASSAYS (Continued)

C. Control Tests

Positive and negative control assays are conducted with each experiment and consist of direct-acting mutagens for nonactivation assays and mutagens that require metabolic biotransformation in activation assays. Negative controls consist of the test compound solvent in the overlay agar together with the other essential components. The negative control plate for each strain gives a reference point to which the test data are compared. The positive control assay is conducted to demonstrate that the test systems are functional with known mutagens.

D. Evaluation Criteria for Ames Assay

Because the procedures used to evaluate the mutagenicity of the test chemical are semiquantitative, the criteria used to determine positive effects are inherently subjective and are based primarily on a historical data base. Most data sets are evaluated using the following criteria:

1. Strains TA-1535, TA-1537, and TA-1538

If the solvent control value is within the normal range, a chemical that produces a positive dose response over three concentrations with the lowest increase equal to twice the solvent control value is considered to be mutagenic.

2. Strains TA-98, TA-100, and D4

If the solvent control value is within the normal range, a chemical that produces a positive dose response over three concentrations with the highest increase equal to twice the solvent control value for TA-100 and two to three times the solvent control value for strains TA-98 and D4 is considered to be mutagenic. For these strains, the dose response increase should start at approximately the solvent control value.

3. Pattern

Because TA-1535 and TA-100 were both derived from the same parental strain (G-46) and because TA-1538 and TA-98 were both derived from the same parental strain (D3052), there is a built-in redundancy in the microbial assay. In general the two strains of a set respond to the same mutagen and such a pattern is sought. It is also anticipated that if a

6. EXPLANATION OF EVALUATION PROCEDURES FOR PLATE ASSAYS (Continued)

D. Evaluation Criteria for Ames Assay

3. Pattern

given strain, e.g. TA-1537, responds to a mutagen in nonactivation tests it will generally do so in activation tests. (The converse of this relationship is not expected.) While similar response patterns are not required for all mutagens, they can be used to enhance the reliability of an evaluation decision.

4. Reproducibility

If a chemical produces a response in a single test that cannot be reproduced in one or more additional runs, the initial positive test data loses significance.

The preceding criteria are not absolute and other extenuating factors may enter into a final evaluation decision. However, these criteria are applied to the majority of situations and are presented to aid those individuals not familiar with this procedure. As the data base is increased, the criteria for evaluation can be more firmly established.

E. Relationship Between Mutagenicity and Carcinogenicity

It must be emphasized that the Ames Salmonella/microsome test is not a definitive test for chemical carcinogens. It is recognized, however, that correlative and functional relationships have been demonstrated between these two end points. The results of comparative tests on 300 chemicals by McCann et al. (Proc. Nat. Acad. Sci. USA, 72:5135-5139, 1975) show an extremely good correlation between results of microbial mutagenesis tests and in vivo rodent carcinogenesis assays.

All evaluation and interpretation of the data presented in this report are based only on the demonstration of or lack of mutagenic activity.

STANDARD OPERATING PROCEDURES

To ensure an accurate and reliable mutagenicity testing program, LBI instituted the following procedures:

- The test compound was registered in a bound log book recording the date of receipt, complete client identification, physical description and LBI code number.
- Complete records of weights and dilutions associated with the testing of the submitted material were entered into a bound notebook.
- Raw data information was recorded on special printed forms that were dated and initialed by the individual performing the data collection at the time the observations were made. These forms were filed as permanent records.
- All animal tissue S-9 preparations used in the activation tests were taken from dated and pretested frozen lots identified by a unique number. The S-9 preparations were monitored for uniformity and the information recorded.