EVALUATION OF POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM IN *SALMONELLA* REVERSE MUTATION ASSAY

FINAL REPORT

IITRI Project Number: 2073-002-001-002
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Sponsor:

Toxicology and Pharmacology Branch
Developmental Therapeutics Program
Division of Cancer Treatment and Diagnosis
National Cancer Institute (NCI)
National Institutes of Health
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NCI Project Officer:

Elizabeth Glaze, Ph.D.

Study Initiation Date: **June 3, 2004**
Study Completion Date: **December 2004**

EVALUATION OF POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM IN *SALMONELLA* REVERSE MUTATION ASSAY

ABSTRACT

The potential mutagenic activity of CuATSM/H_2ATSM was investigated in the *Salmonella* reverse mutation assay. The plate incorporation method was employed using *Salmonella typhimurium* tester strains TA98, TA100, TA102, TA1535, and TA1537, in the presence and absence of an external metabolic activation system (i.e., Aroclor 1254-induced rat liver S9 fraction mixture).

In the initial mutagenicity/cytotoxicity assay, the dose levels of CuATSM/H_2ATSM were 10, 25, 30, 40, and 100 μg/plate and were selected by expected solubility limits. CuATSM/H_2ATSM did not exhibit a dose-related mutagenic response in any of the five tester strains either with or without S9 metabolic activation. Additionally, there was no evident cytotoxicity observed for any strain in the presence or absence of S9. There was no visibly apparent precipitation at dose level up to 100 μg/plate.

Because no visibly apparent precipitate was observed in the initial mutagenicity/cytotoxicity assay, the dose range was increased to include 500 and 1,000 μg/plate in the confirmatory mutagenicity assay. At these dose levels precipitation of CuATSM/H_2ATSM was visually apparent and precipitation was microscopically confirmed at the 100 μg/plate dose level. Generally, there was no dose-related mutagenic response in any of the five tester strains either in the presence or absence of S9. In the confirmatory mutagenicity assay, at the 1,000 μg/plate dose level with strain TA100 in the absence of S9 there was approximately a two-fold increase of revertants relative to the vehicle control. This increase, however, was not dose-responsive in the test group and was only observed at the 1,000 μg/plate dose level which was over 10 times the limit of solubility of the CuATSM/H_2ATSM. In view of the lack of a dose-responsive trend and the observation of increased revertant colonies at only one dose level that was far in excess of the limit of solubility, it is likely that the mutagenic response observed in the absence of S9 in strain TA100 was a result of the presence of soluble impurities in the CuATSM/H_2ATSM preparation rather than a result of treatment with the CuATSM/H_2ATSM itself.

The results of this study indicate that CuATSM/H_2ATSM is negative for cytotoxicity and mutagenicity in the *Salmonella* reverse mutation assay. Although there was a response with strain TA100 at the 1,000 μg/plate dose level, this observation was concluded to result from the

presence of soluble impurities in the CuATSM/H_2ATSM preparation and not from the CuATSM/H_2ATSM.

EVALUATION OF POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM IN *SALMONELLA* REVERSE MUTATION ASSAY

COMPREHENSIVE SUMMARY

Title:	Evaluation of Potential Mutagenic Activity of CuATSM/H_2ATSM in *Salmonella* Reverse Mutation Assay
Strain:	*Salmonella* typhimurium TA98, TA100, TA102, TA1535 and TA1537
Dose Levels:	Five dose levels for each tester strain were tested for the ability to induce reverse mutations. Initial mutagenicity/cytotoxicity assay – 10, 25, 30, 40 and 100 µg/plate, with and without S9 Confirmatory mutagenicity assay – 10, 40, 100, 500 and 1000 µg/plate with and without S9
Test Article:	CuATSM/H_2ATSM [Copper-Diacetyl-bis(N^4-methylthiosemicarbazone)/Diacetyl-bis(N^4-methylthiosemicarbazone)]
Control Article:	Dimethylsulfoxide (DMSO)

RESULTS:

Mutagenicity/Cytotoxicity Assay:	In the initial mutagenicity/cytotoxicity assay there was no dose-related mutagenic response in any of the five tester strains either with or without S9 metabolic activation. Additionally, there was no evident cytotoxicity observed for any strain in the presence or absence of S9. In the confirmatory mutagenicity assay there was no clear cytotoxicity observed up to 1,000 µg/plate for any strain in the presence or absence of S9 metabolic activation. Generally, there was no dose-related mutagenic response in any of the five tester strains either in the presence or absence of S9. However, CuATSM/H_2ATSM did demonstrate a mutagenic response in strain TA100 in the absence of S9 at a dose level of 1,000 µg/plate.
CONCLUSIONS:	CuATSM/H_2ATSM was not toxic to *Salmonella typhimurium* tester strains TA98, TA100, TA102, TA1535, and TA1537 and it was negative under the conditions tested in the *Salmonella* reverse mutation assay. Although there was a response with strain TA100 at the 1,000 µg/plate dose level, this observation was concluded to result from the presence of soluble impurities in the CuATSM/H_2ATSM preparation and not from the CuATSM/H_2ATSM.

EVALUATION OF POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM IN *SALMONELLA* REVERSE MUTATION ASSAY

Study Initiation Date: June 3, 2004
Experimental Initiation Date: July 2, 2004
Experimental Termination Date: July 12, 2004

FOREWORD

This report describes a *Salmonella* reverse mutation assay conducted by IIT Research Institute (IITRI) for the National Cancer Institute (NCI). The NCI Project Officer for the study was Elizabeth Glaze, Ph.D.

Nei-Long Lyang, M.S., C.B.S., served as Study Director and was responsible for the overall conduct of the study. David L. McCormick, Ph.D., D.A.B.T., Vice President and Director, Life Sciences Group, served as Principal Investigator. Glenn B. Miller, M.S., C.Q.M., Manager, Quality Assurance, was responsible for the IITRI quality assurance program.

Nei-Long Lyang 12/7/04

Nei-Long Lyang, M.S., C.B.S. Date
Study Director
Life Sciences Group

12/7/04

David L. McCormick, Ph.D., D.A.B.T. Date
Principal Investigator
Vice President and Director
Life Sciences Group

EVALUATION OF POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM IN *SALMONELLA* REVERSE MUTATION ASSAY

GLP COMPLIANCE STATEMENT

This study was conducted in compliance with the U.S. Food and Drug Administration (FDA) Good Laboratory Practice Regulations (*Code of Federal Regulations* Title 21 Part 58). The study raw data have been reviewed and the information contained in this report is an accurate representation of the data within the context of the study design and evaluation criteria.

Nei-Long Lyang 12/7/04

Nei-Long Lyang, M.S., C.B.S. Date
Study Director
Life Sciences Group

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EVALUATION OF POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM IN *SALMONELLA* REVERSE MUTATION ASSAY

I. INTRODUCTION

The *Salmonella* reverse mutation assay is an *in vitro* test designed to detect point mutations in bacterial tester strains induced by chemical agents. The mutagenic events are reverse mutations that cause histidine-requiring mutants to revert to their prototrophic (non-histidine requiring) state. The objective of this study was to determine the potential mutagenic activity of CuATSM/H_2ATSM using the *Salmonella* reverse mutation assay with and without metabolic activation.

II. MATERIALS AND METHODS

A copy of the study protocol is presented in Appendix A.

A. Test and Control Articles: The test article, CuATSM/H_2ATSM [Copper-Diacetyl-bis(N^4-methylthiosemicarbazone)/Diacetyl-bis(N^4-methylthiosemicarbazone)]; Galbraith Laboratories Inc., Knoxville, TN), a blue-gray powder, was received on October 9, 2003 in an amber glass vial and was stored at room temperature (approximately 20-25°C). The identity, strength, quality, stability and purity, as well as documentation of methods of synthesis, fabrication or derivation of the bulk test article, were the responsibility of the Sponsor. A CuATSM/H_2ATSM certificate of analysis is included in Appendix D.

The control article was Dimethylsulfoxide (DMSO). The control article, a clear, colorless liquid, was stored at room temperature (approximately 20-25°C). A certificate of analysis for the control article (from Sigma-Aldrich, St. Louis, MO) is included in Appendix D.

B. Test Article Formulation: For the initial mutagenicity/cytotoxicity assay, a 1,000 µg/mL (w/v) test article stock solution was prepared by mixing 0.010 g of CuATSM/H_2ATSM with 10 mL (*q.s.*) of DMSO. For the confirmatory mutagenicity assay, a 10,000 µg/ml (w/v) test article stock solution was prepared by mixing 0.07 g of CuATSM/H_2ATSM with 7 ml (q.s.) of DMSO. From this stock solution, test article formulations were prepared by adding appropriate volumes of the vehicle to the CuATSM/H_2ATSM stock solution. The stock solution and test article formulations were prepared and assayed on July 2 and 9, 2004.

C. Experimental Design and Procedures

1. Media and Reagents Preparation: All media and reagents were prepared using purified distilled ATSM Type 1 water. Glassware, media, and solutions were sterilized either by autoclaving or by passing through 0.22 μm filters. All chemicals were obtained from Sigma Chemical Company, St. Louis, MO or Fisher-Acros, Pittsburgh, PA, unless otherwise indicated.

For bacterial cultures, Nutrient Broth No. 2 from OXOID (Hampshire, England) was used at a concentration of 25 g/l. Culture plates were prepared with minimal glucose agar at 20 ml/plate using a bench-top autoclave (AGARCLAV, Integra Biosciences GmbH, Fernwald, Germany) and an automatic dispensing and stacking unit (TECNOMAT, Integra Biosciences GmbH). The minimal glucose agar consisted of 930 ml of ATSM Type 1 water per liter of media, 15 g/l agar (OXOID), 20 g/l glucose (Difco, Detroit, MI), and 20 ml Vogel-Bonner Medium E (VBE) salts (50X) with 0.81 mmol/l $MgSO_4 \cdot 7H_2O$, 9.52 mmol/l citric acid monohydrate, 57.4 mmol/l K_2HPO_4, and 16.7 mmol/l $Na(NH_4)HPO_4 \cdot 4H_2O$. The glucose was filter-sterilized. The VBE salts and the agar with water were sterilized separately. Condensation of excess water on the solid agar surface was prevented by keeping the plates overnight in an inverted position at room temperature. The plates were stored at approximately 4°C in a walk-in refrigerator for a maximum period of 3 months until use.

The standard plate incorporation method was used to plate the bacteria along with the test article and the S9 metabolic activation system or buffer. The top agar consisted of 5.5 g/l agar (OXOID), 85.6 mmol/l NaCl, 0.05 mmol/l L-histidine, and 0.05 mmol/l D-biotin. Molten agar-NaCl solution was autoclaved and allowed to cool to approximately 55°C, after which 10 ml aliquots of filter-sterilized L-histidine/D-biotin solution were added to each 100 ml of agar-NaCl solution. Finally, the agar was dispensed into test tubes (2 ml per tube) and stored at approximately 55°C until used.

For metabolic activation of promutagens, Aroclor 1254-induced rat liver post-mitochondrial S9 fraction (Lot Number 1659, Molecular Toxicology Inc., Boone, NC) was used. Five hundred microliters (500 μl) of a 10% (v/v) S9 mixture was added to 2 ml of soft agar per plate. The 10% (v/v) S9 mixture consisted of 100 mmol/l sodium phosphate buffer (pH 7.4), 8 mmol/l $MgCl_2 \cdot 6H_2O$, 33 mmol/l KCl, 5 mmol/l glucose-6-phosphate monosodium, and 4 mmol/l NADP. The S9 mixture

was filter sterilized by 0.45 μm syringe filter prior to use and kept in the ice bath during the experiment.

2. Selection of Test System Justification: The *Salmonella* reverse mutation assay is an *in vitro* test designed to detect point mutations in bacterial tester strains induced by chemical agents. This test has been approved by regulatory agencies in the United States and abroad (EPA Guideline OPPTS 870.5100 and OECD Guideline 471) to detect the mutagenic potential of chemicals, drugs and environmental agents.

3. Test System and Procedure: The *Salmonella* reverse mutation assay was conducted in accordance with the methods described by Maron and Ames (1983). In this test, the plate incorporation method was used to detect the mutagenic potential of CuATSM/H_2ATSM. Duplicate runs of the assay were performed on July 2, 2004 (Initial) and July 9, 2004 (Confirmatory). The assay was conducted in compliance with procedures identified by the U.S. Food and Drug Administration (FDA) in the *FDA Redbook 2000*.

   Five different *Salmonella typhimurium* bacterial tester strains were used in this assay. Four mutant tester strains that detect histidine reversion at G-C sites were obtained from Molecular Toxicology, Boone, NC (TA98, TA1535, and TA1537) or from Xenometrix, San Diego, CA (TA100). Tester strain TA102, used to enhance the capability of detecting oxidizing mutagens and hydrazines, was also obtained from Xenometrix. All strains have a rfa (deep rough) mutation that eliminates the polysaccharide side chain of the lipopolysaccharide (LPS) layer coating the bacterial surface, thereby increasing permeability to larger molecules. Four strains (TA98, TA100, TA1535 and TA1537) also have a mutation that is the deletion of a gene coding for the DNA excision repair system (*uvrB*), which increases the sensitivity of the bacteria to mutagens. Three strains (TA98, TA100 and TA102) contain the R-factor plasmid (pKM101) and are ampicillin resistant. In addition, strain TA102 contains the *hisG428* mutation on the multi-copy plasmid (pAQ1), which carries a tetracycline-resistant gene. All five bacterial strains have a histidine requirement that allows for the detection of revertants on minimal medium plates. A summary of mutations of tester strains is described in the following table:

Strain Designation	Histidine Mutation	Additional Mutations			Mutation-type Detection
		LPS	Repair	R-factor	
TA98	*HisD3052*	Rfa	*uvrB*	pKM 101	Frameshift
TA100	*HisG46*	Rfa	*uvrB*	pKM 101	Base-pair substitution
TA102	*HisG428*	Rfa	Wild Type	pKM 101	Ochre
TA1535	*HisG46*	Rfa	*uvrB*	-	Base-pair substitution
TA1537	*HisC3076*	Rfa	*uvrB*	-	Frameshift

Phenotypic and genotypic characterization of the bacterial cultures used in this study was performed on July 19-22, 2004. The results of that confirmatory assay are presented in Appendix C Table C-1. The bacteria were maintained as stock cultures in Nutrient Broth No. 2 (OXOID; with 25 μg/ml ampicillin for TA 98 and TA100, with 25 μg/ml ampicillin and 2 μg/ml tetracycline for TA102) with DMSO in liquid nitrogen. One day before the assay, the tester strain bacteria were cultivated in 20 ml of nutrient broth with approximately 1.0 ml of the thawed stock culture in duplicate. The bacteria were incubated at 37±1°C on an orbital shaker at a speed of 100 to 120 rpm for 15±2 hours to obtain logarithmic/early stationary growth phase cultures. The optical density (O.D.) of the overnight culture was determined at 650 nm. After obtaining the O.D., ten-fold serial dilutions were made and the bacteria of each strain were grown on brain heart infusion agar (BHIA) plates. After an 18- to 24-hour incubation period at 37±1°C, the colonies on BHIA plates were counted. The numbers of viable cells are usually found to give rise to a minimum of $1.0x10^8$ cfu/ml. The viabilities of the cultures used in each batch were confirmed and results are presented in Appendix C Table C-2.

On the day of the assay, 100 μl of the appropriate dilution of test article, 100 μl bacterial culture, and 500 μl (10%, v/v) S9 mixture (or sodium phosphate buffer, pH 7.4, for plates without S9), were added to 2 ml of top agar. The components were vortexed for about 3 seconds at low to mid speed, and poured and spread evenly on minimal glucose agar plates. After the top agar solidified, the plates were inverted and incubated at 37±1°C for between 48 and 72 hours. The number of revertant colonies in each plate was counted in an AccuCount 1000 Automatic Colony Counter (Biologics, Inc., Gainesville, VA).

For each bacterial strain and activation condition (with or without S9), three plates for each of the solvent/negative control, positive control, and test article were

prepared. Each plate was identified with a label indicating the project number, dose of the test article (or positive or solvent/negative control), metabolic activation system, individual plate number, tester strain, and the assay date.

In addition to positive and solvent/negative controls, sterility controls were run simultaneously with the test article. Sterility control plates were of two types. The type I plates were intended to test for microbial contaminants in the test article. The test consisted of a single plate per dose comprised of the test article, the overlay top agar, and the VBE minimal glucose medium. The type II sterility plates tested for microbial contaminants in the S9 mixture or sodium phosphate buffer. It consisted of triplicate plates of overlay top agar, S9 mixture or sodium phosphate buffer, and the VBE minimal glucose medium.

4. Positive Control Chemicals: To confirm sensitivity of the test organisms, a number of positive control mutagens were tested in this assay. These included 2-Aminoanthracene (2-AA), Cumene Hydroperoxide, Daunomycin and ICR-191 (all from Sigma Chemical Company, St. Louis, MO), as well as Danthron, Methyl Methanesulfonate (MMS) and Sodium Azide (NaN_3; Fisher-Acros, Pittsburgh, PA) and 2-Aminofluorene (2-AF; Fluka, St. Louis, MO). The following table describes the list of chemicals tested in each strain of bacteria with or without metabolic activation using S9, which was purchased from Molecular Toxicology Inc., Boone, NC:

Strain	With S9	Without S9
TA98	2-AA, 2-AF	Daunomycin
TA100	2-AA, 2-AF	MMS
TA102	Danthron	Cumene Hydroperoxide
TA1535	2-AA	Sodium Azide
TA1537	2-AA	ICR-191

As part of each batch assay, confirmatory negative and positive control assays were performed on July 2, 2004 (Initial) and July 9, 2004 (Confirmatory). These results are presented in Appendix B Tables B-11 and B-12.

D. Data Interpretation and Results: Data were presented as the number of revertants/plate along with mean and standard deviation (SD). The plate count was expressed as number of revertants/plate per concentration of test article.

E. Archives: All data documenting experimental details and study procedures will be recorded and maintained as raw data. At the completion of the study, all raw data generated at IITRI and a copy of the final report will be maintained in the IITRI archives for a period of one year after submission of the signed final report. At that time, the Sponsor will be contacted in order to determine the final disposition of the archival materials. IITRI's Quality Assurance Unit will maintain a complete record of the disposition of all archival materials.

III. RESULTS

A. Organism Characterization: Results of the genotypic and phenotypic characterization of the bacterial tester strains, performed July 19-22, 2004 are presented in Appendix C Table C-1, and results of the viability assays (Initial and Confirmatory) are presented in Appendix C Table C-2. These results indicate that the genotypes of all strains were intact and viable when tested.

Mutagenic response (as measured by number of revertant colonies per plate) of tester strains to various positive control chemicals (both S9-dependent and S9-independent) are presented in Appendix B Table B-11 (Initial) and B-12 (Confirmatory). Responses to the positive control chemicals presented at least a 2-fold increase over the vehicle/solvent control, indicating that the tester strains were responsive to the presence of mutagenic agents. Also, in both assays with and without S9, tester strain responses to the vehicle/solvent control and to the positive control mutagens (i.e., 2-AA, 2-AF, Danthron, Daunomycin, Cumene Hydroperoxide, ICR-191, MMS and Sodium Azide) were within historical limits. The IITRI historical data for positive and vehicle/solvent control responses is presented in Appendix C Table C-3.

B. Mutagenicity Assay Results: The means of revertants per plate per CuATSM/H_2ATSM concentration are summarized in Tables 1 and 2. The means of revertants per plate are also depicted graphically in Figures 1 through 5. Individual assay results are presented in Appendix B Tables 1 through 10.

In the initial mutagenicity/cytotoxicity assay, CuATSM/H_2ATSM (at dose levels of 10, 25, 30, 40, and 100 μg/plate) did not exhibit a dose-related mutagenic response in any of the five tester strains either with or without S9 metabolic activation. Additionally, there was no evident cytotoxicity observed for any strain in the presence or absence of S9. In

this mutagenicity/cytotoxicity assay, there was no visibly apparent precipitation at dose level up to 100 μg/plate.

According to the mutagenicity and cytotoxicity results observed in the initial mutagenicity/cytotoxicity assay, the dose range of CuATSM/H_2ATSM was increased to include 500 and 1,000 μg/plate in the confirmatory mutagenicity assay. The results indicated that CuATSM/H_2ATSM did not exhibit cytotoxicity up to 1,000 μg/plate for any strain in the presence or absence of S9 metabolic activation. Similar to the initial mutagenicity/cytotoxicity assay, there was no dose-related mutagenic response in any of the five tester strains either in the presence or absence of S9 in the confirmatory mutagenicity assay. However, CuATSM/H_2ATSM did demonstrate a mutagenic response in strain TA100 in the absence of S9 at a dose level of 1,000 μg/plate. For this observation, the maximum number of revertants per plate over the number of spontaneous revertants (i.e., solvent/negative control, DMSO) was scarcely over 2-fold. In addition, precipitation of CuATSM/H_2ATSM was seen at dose levels of 500 and 1,000 μg/plate; CuATSM/H_2ATSM at dose level of 100 μg/plate showed no visibly apparent precipitate, however, the presence of precipitate was determined microscopically.

IV. DISCUSSION AND CONCLUSIONS

There are several criteria for determining a positive result, such as a dose-related increase over the range tested and a reproducible increase at one or more doses in the number of revertant colonies per plate in at least one strain with or without metabolic activation system (*FDA, Redbook 2000*). A 2-fold increase in the number of revertant colonies per plate, when compared to that of vehicle/solvent control, was observed at the 1,000 μg/plate dose level with strain TA100 in the absence of S9 metabolic activation. This increase, however, was not dose-responsive in the test group and was only observed at the 1,000 μg/plate dose level which was over 10 times the limit of solubility of the CuATSM/H_2ATSM. In view of the lack of a dose-responsive trend and the observation of increased revertant colonies at only one dose level that was far in excess of the limit of solubility, it is likely that the mutagenic response observed in the absence of S9 in strain TA100 was a result of the presence of soluble impurities in the CuATSM/H_2ATSM preparation rather than a result of treatment with the CuATSM/H_2ATSM itself.

The results of this study indicate that CuATSM/H_2ATSM is negative for cytotoxicity and mutagenicity in the *Salmonella* reverse mutation assay. Although there was a response with strain TA100 at the 1,000 μg/plate dose level, this observation was concluded to result from the presence of soluble impurities in the CuATSM/H_2ATSM preparation and not from the CuATSM/H_2ATSM.

V. REFERENCES

a. FDA Redbook 2000, Toxicological principles for the safety of food ingredients: IV.C.1.a Bacterial reverse mutation test, July 7, 2000

b. Maron, D.M. and Ames, B.N. 1983. Revised methods for the *Salmonella* mutagenicity test. Mutation Research. 113:173-215.

VI. QUALITY ASSURANCE STATEMENT

Study Title: Evaluation of Potential Mutagenic Activity of CuATSM/H_2ATSM in *Salmonella* Reverse Mutation Assay

Project Number: 2073-002-001-002

Study Director: Nei-Long Lyang, M.S., C.B.S.

This study has been inspected and the report has been audited by the IITRI Quality Assurance Unit in accordance with the Food and Drug Administration's "Good Laboratory Practice Regulations" – "*Code of Federal Regulations* Title 21 Section 58.35." The report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

The following are the inspection dates and the dates inspection findings were reported:

	Findings Reported To:	
Inspection Dates	Study Director	Management
May 19, 2004	May 19, 2004	May 20, 2004
July 2, 2004	July 6, 2004	July 9, 2004
July 9, 2004	July 9, 2004	July 20, 2004
October 11, 2004	October 12, 2004	October 15, 2004

Glenn B. Miller, M.S., C.Q.M.
Manager, Quality Assurance

12-3-04
Date

VII. TABLES

EVALUATION OF POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM IN *SALMONELLA* REVERSE MUTATION ASSAY

Table 1

Summary of the Mutagenicity/Cytotoxicity of CuATSM/H_2ATSM in *Salmonella* Tester Strains with and without S9 – Initial Assay

		REVERTANTS/PLATE (Mean[a] ± SD)				
		STRAIN				
S9	DOSE ATTM (µg/plate)	TA98	TA100	TA102	TA1535	TA1537
+	0	24 ± 6.7	116 ± 5.3	244 ± 31.1	9 ± 2.1	2 ± 1.7
+	10	26 ± 2.5	125 ± 11.1	270 ± 43.8	8 ± 1.5	4 ± 2.9
+	25	30 ± 5.5	111 ± 14.5	276 ± 23.7	12 ± 1.5	4 ± 1.7
+	30	27 ± 5.5	120 ± 7.5	252 ± 28.2	11 ± 4.7	4 ± 1.5
+	40	31 ± 7.5	134 ± 7.5	283 ± 22.6	10 ± 3.6	3 ± 1.5
+	100	32 ± 9.6	129 ± 6.5	275 ± 30.3	8 ± 2.6	3 ± 0.6
-	0	19 ± 4.0	111 ± 2.5	202 ± 12.3	6 ± 1.2	5 ± 0.0
-	10	14 ± 4.7	113 ± 9.1	173 ± 24.0	14 ± 4.0	2 ± 1.5
-	25	18 ± 2.5	112 ± 7.1	195 ± 2.6	10 ± 2.1	3 ± 1.0
-	30	20 ± 7.2	105 ± 7.0	228 ± 0.6	9 ± 3.1	5 ± 3.5
-	40	19 ± 3.6	118 ± 6.7	223 ± 5.1	10 ± 3.6	4 ± 1.2
-	100	22 ± 2.5	117 ± 4.6	226 ± 16.5	13 ± 4.0	5 ± 3.0

DATE OF ASSAY: 07-02-2004 (Initial)

[a]Per dose, N = 3

EVALUATION OF POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM IN *SALMONELLA* REVERSE MUTATION ASSAY

Table 2

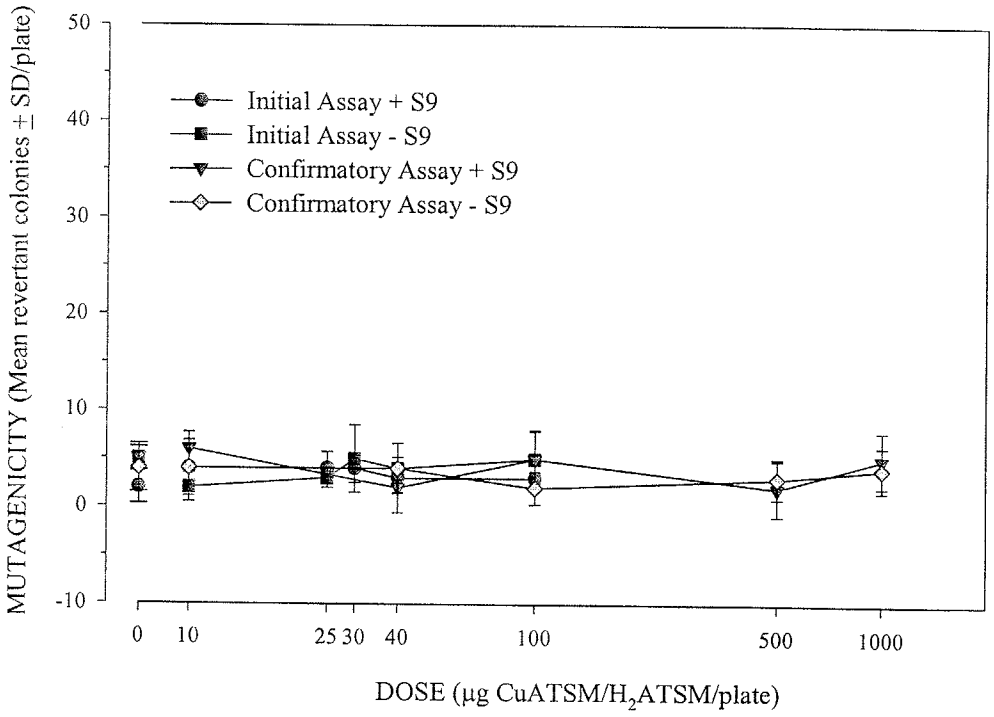
Summary of the Mutagenicity/Cytotoxicity of CuATSM/H_2ATSM in *Salmonella* Tester Strains with and without S9 – Confirmatory Assay

		REVERTANTS/PLATE (Mean[a] ± SD)				
		STRAIN				
S9	DOSE ATTM (µg/plate)	TA98	TA100	TA102	TA1535	TA1537
+	0	28 ± 5.5	122 ± 16.8	342 ± 25.1	10 ± 1.2	5 ± 1.2
+	10	27 ± 4.6	149 ± 22.6	296 ± 38.1	8 ± 2.1	6 ± 1.7
+	40	25 ± 7.4	126 ± 12.6	264 ± 42.0	12 ± 2.5	2 ± 2.6
+	100	28 ± 8.7	121 ± 21.8	256 ± 39.0	10 ± 0.6	5 ± 2.9
+	500	30 ± 5.9	136 ± 11.1	322 ± 22.6	14 ± 7.5	2 ± 2.9
+	1000	30 ± 6.2	125 ± 8.7	345 ± 10.1	16 ± 3.5	5 ± 2.9
-	0	17 ± 3.2	102 ± 8.7	241 ± 7.4	14 ± 1.5	4 ± 2.5
-	10	20 ± 1.5	114 ± 6.1	222 ± 39.7	16 ± 6.1	4 ± 2.3
-	40	16 ± 4.7	106 ± 6.2	245 ± 28.0	10 ± 2.1	4 ± 2.6
-	100	13 ± 3.0	108 ± 22.6	252 ± 14.4	6 ± 2.3	2 ± 1.7
-	500	26 ± 5.5	143 ± 13.9	267 ± 17.1	21 ± 5.5	3 ± 2.1
-	1000	23 ± 3.1	231 ± 66.2	395 ± 47.6	14 ± 1.2	4 ± 2.3

DATE OF ASSAY: 07-09-2004 (Confirmatory)
[a]Per dose, N = 3

Figure 5

Mutagenicity (Mean revertants/plate) of CuATSM/H_2ATSM (Microgram/plate) for Strain TA1537 with and without S9 in Both Assays



NOTE: Log Scale Applied for Doses of CuATSM/H_2ATSM;
Vehicle/Solvent Control at 0 Dose.

IX. APPENDICES

Appendix A. Protocol

Appendix A

EVALUATION OF POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM IN *SALMONELLA* REVERSE MUTATION ASSAY

SPONSOR: Toxicology & Pharmacology Branch
Developmental Therapeutics Program
Division of Cancer Treatment and Diagnosis
National Cancer Institute (NCI)
National Institutes of Health
Bethesda, Maryland 20892

PROJECT OFFICER: Elizabeth Glaze, Ph.D.

CONTRACT NUMBER: N01-CM-42202

CONTRACTOR: IIT Research Institute
10 West 35th Street
Chicago, IL 60616-3799

PRINCIPAL INVESTIGATOR: David L. McCormick, Ph.D., D.A.B.T.

STUDY DIRECTOR: Nei-Long Lyang, M.S., C.B.S.

PROPOSED EXPERIMENTAL PHASE:

Start: July 1, 2004

Finish: July 23, 2004

I. OBJECTIVE:

The objective of this study is to determine the potential mutagenic activity of CuATSM/H_2ATSM using the *Salmonella* reverse mutation assay with and without metabolic activation.

II. MATERIALS AND METHODS:

A. Test and Control Articles:

1. Name of Test Article:

CuATSM/H_2ATSM
[Copper-Diacetyl-bis(N^4-methylthiosemicarbazone)/Diacetyl-bis(N^4-methylthiosemicarbazone)]

2. Name of Vehicle Control:

Dimethylsulfoxide (DMSO).

3. Characterization and Documentation of Methods of Synthesis, Fabrication or Derivation:

a. Test Article:

Compound identity, strength, quality, stability, and purity as well as documentation of methods of synthesis, fabrication or derivation are the responsibility of NCI.

b. Vehicle Control:

Characterization of DMSO may be attained by the certificate of analysis, by recording all pertinent information provided on the container labels, or by retaining the container labels themselves as raw data.

4. Stability and Storage:

a. Test Article:

The test article is stored at room temperature.

b. Vehicle Control:

DMSO is stable until the expiration date specified by the manufacturer, when stored in accordance with the label instructions.

5. Formulation Preparation, Stability and Storage:

The test article is stable in DMSO for the duration of the study, when stored at approximately -70°C.

B. Test System:

1. Species, Strain, Supplier and Test System Justification:

The *Salmonella* reverse mutation assay is an *in vitro* test designed to detect point mutations in bacterial tester strains induced by chemical agents. Five different strains of *Salmonella typhimurium* histidine auxotrophs TA98, TA100, TA102, TA1535 and TA1537 will be used in this study. The sources of the strains used in the study will be documented. These strains are routinely used to evaluate the preclinical toxicity (mutagenicity) of compounds used, or intended for use, in humans (FDA Redbook 2000).

C. Experimental Design:

The mutagenicity of test article, CuATSM/H_2ATSM, will be determined in five different *Salmonella typhimurium* tester strains with and without metabolic activation (S9). Appropriate positive controls and solvent/vehicle control (DMSO) will be included while testing the mutagenicity of the test article.

1. Mutagenicity Assay Procedures:

a. Media and Reagent Preparation:

All media and reagents will be prepared using purified distilled ASTM type1 water. Glassware, media, and solutions will be sterilized either by autoclaving or by passing through 0.22 µM filters. All chemicals will be obtained from Sigma, St. Louis, MO, or Fisher-Acros, Pittsburgh, PA, unless otherwise indicated.

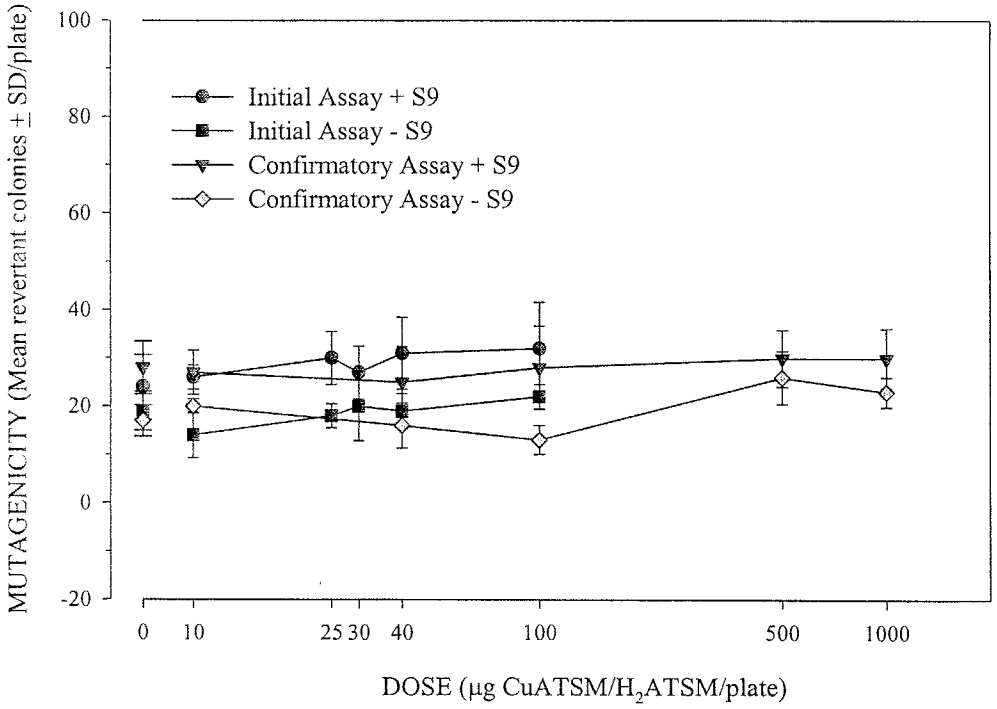
For bacterial cultures, Nutrient Broth No. 2 from OXOID will be used at a concentration of 25 g/l. Culture plates will be prepared with minimal glucose agar at 20 ml/plate using a bench top autoclave (AGARCLAV, Integra Biosciences GmbH, Fernwald, Germany) and an automatic dispensing and stacking unit (TECNOMAT, Integra Biosciences GmbH, Fernwald, Germany). The minimal glucose agar consists of 15 g/l agar (Oxoid, Hampshire, England) in ASTM Type 1 water, 20 g/l glucose, and Vogel-Bonner

VIII. FIGURES

EVALUATION OF POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM IN *SALMONELLA* REVERSE MUTATION ASSAY

Figure 1

Mutagenicity (Mean revertants/plate) of CuATSM/H_2ATSM (Microgram/plate) for Strain TA98 with and without S9 in Both Assays

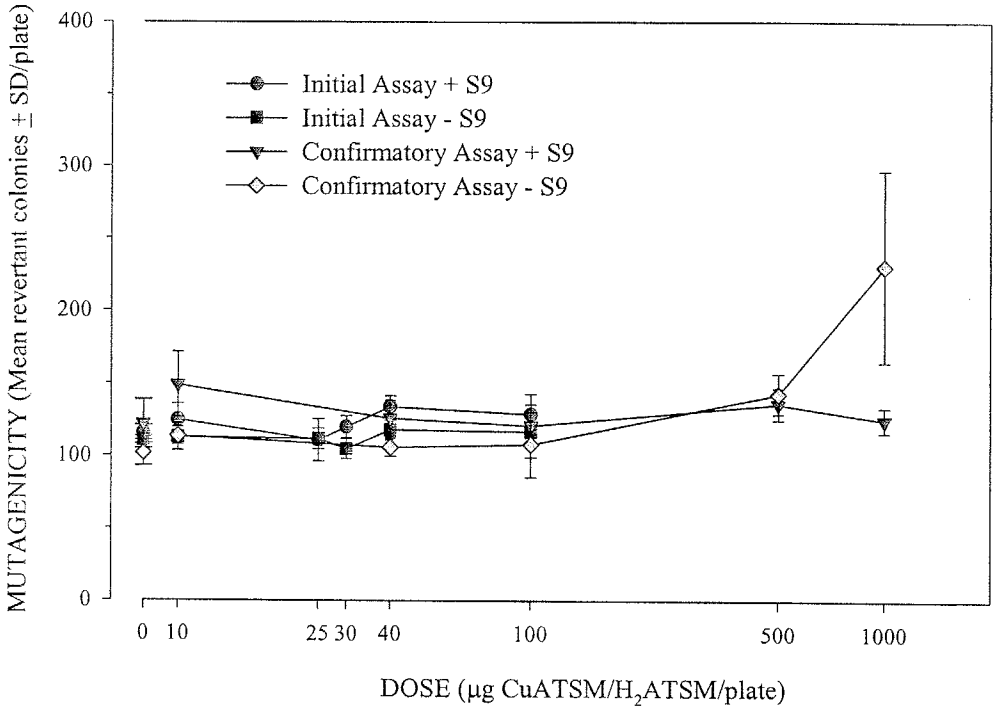


NOTE: Log Scale Applied for Doses of CuATSM/H_2ATSM;
Vehicle/Solvent Control at 0 Dose.

EVALUATION OF POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM IN *SALMONELLA* REVERSE MUTATION ASSAY

Figure 2

Mutagenicity (Mean revertants/plate) of CuATSM/H_2ATSM (Microgram/plate) for Strain TA100 with and without S9 in Both Assays

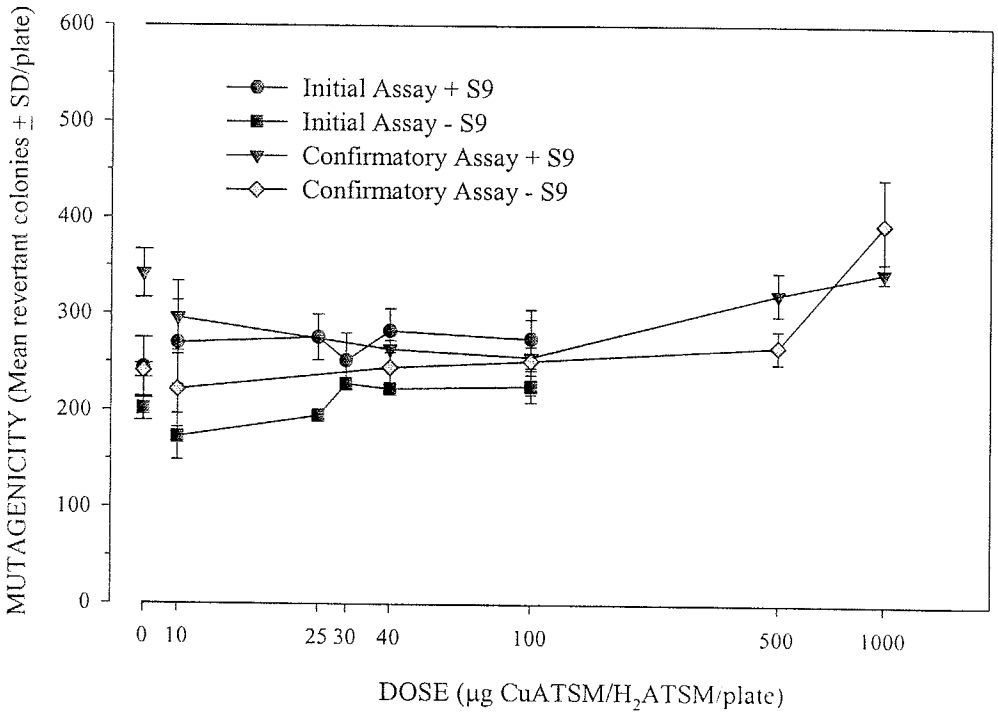


NOTE: Log Scale Applied for Doses of CuATSM/H_2ATSM;
Vehicle/Solvent Control at 0 Dose.

EVALUATION OF POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM IN *SALMONELLA* REVERSE MUTATION ASSAY

Figure 3

Mutagenicity (Mean revertants/plate) of CuATSM/H_2ATSM (Microgram/plate) for Strain TA102 with and without S9 in Both Assays

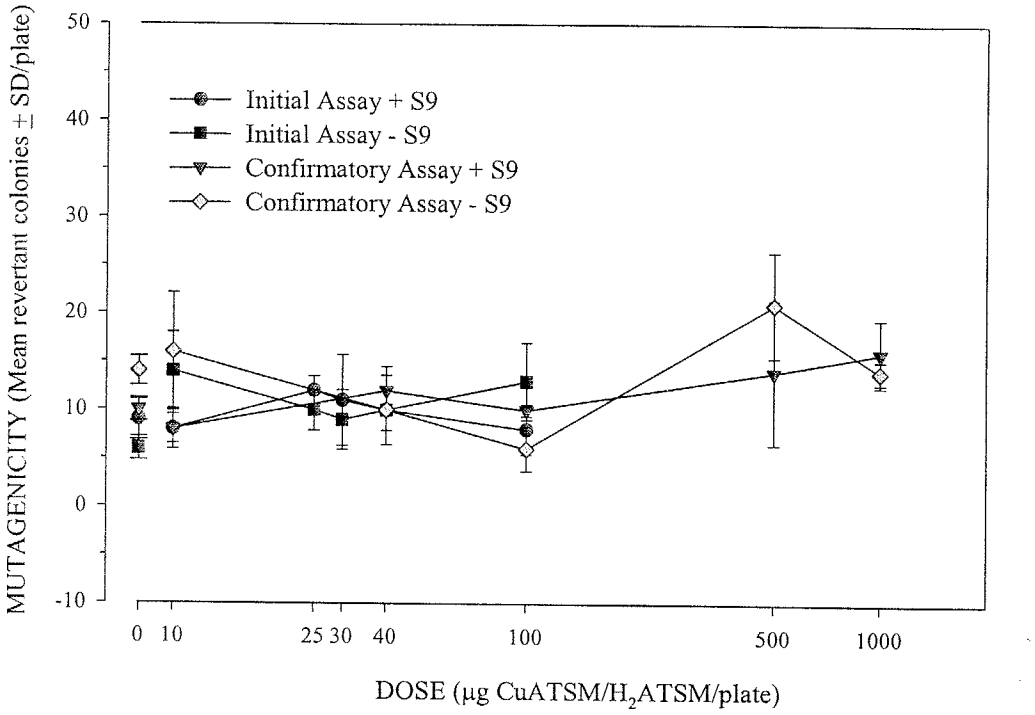


NOTE: Log Scale Applied for Doses of CuATSM/H_2ATSM; Vehicle/Solvent Control at 0 Dose.

EVALUATION OF POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM IN *SALMONELLA* REVERSE MUTATION ASSAY

Figure 4

Mutagenicity (Mean revertants/plate) of CuATSM/H_2ATSM (Microgram/plate) for Strain TA1535 with and without S9 in Both Assays



NOTE: Log Scale Applied for Doses of CuATSM/H_2ATSM;
Vehicle/Solvent Control at 0 Dose.

medium E salts (VBE Salts 50X, Molecular Toxicology, Boone, NC) with 0.81 mmol/l $MgSO_4 \cdot 7H_2O$, 9.52 mmol/l citric acid monohydrate, 57.4 mmol/l K_2HPO_4, and 16.7 mmol/l $Na(NH_4)HPO_4 \cdot 4H_2O$. The glucose will be filter sterilized. The VBE salts (50X) and the agar/water mixture will be sterilized separately. After the plates are prepared, the excess water from the solid agar surface will be removed by keeping the plates in an inverted position overnight at room temperature. The plates will be stored at approximately 4°C in a walk-in refrigerator until use for a maximum period of 3 months.

The standard plate incorporation method will be used to plate bacteria along with the test article and the S9 metabolic activation mixture or buffer. The top agar will consist of 5.5 g/l agar (Oxoid), 85.6 mmol/l NaCl, 0.05 mmol/l L-histidine, and 0.05 mmol/l D-biotin. Molten agar-NaCl solution will be autoclaved, and allowed to cool to approximately 55°C. Then, filter sterilized L-histidine/D-biotin solution will be added in 10 ml aliquots per 100 ml of molten agar-NaCl solution. Finally, the molten top agar will be dispensed into test tube (2 ml/tube), and will be kept at approximately 55°C until used.

For metabolic activation of promutagens, Aroclor-1254-induced rat liver post-mitochondrial S9 fraction (Molecular Toxicology Inc., Boone, NC) will be used. The 10% (v/v) S9 mixture will consist of 100 mmol/l sodium phosphate buffer, pH 7.4, 8 mmol/l $MgCl_2 \cdot 6H_2O$, 33 mmol/l KCl, 5 mmol/l glucose-6-phosphate monosodium, and 4 mmol/l NADP. The S9 mixture will be filter sterilized by 0.45 μm syringe filter prior to use, and kept in an ice bath during the experiment.

b. Test System and Procedure:

The *Salmonella* reverse mutation assay will be conducted in accordance with the methods described by Maron and Ames (1983). In this test, the plate incorporation method will be used to detect the mutagenic potential of test article. The assay will be conducted in compliance with procedures identified by the U.S. Food and Drug Administration (FDA Redbook 2000).

Five different bacterial strains will be used in this assay. *Salmonella typhimurium* tester strains - TA98 and TA100, TA1535 and TA1537 detect histidine *(his)* reversion *(his*$^-$ → *his*$^+$) at G-C sites. To enhance the capability of detecting oxidizing mutagens and hydrazines, strain TA 102 will be also used. All strains have an rfa (deep rough) mutation that eliminates the polysaccharide side chain of the lipopolysaccharide (LPS) layer coating the bacterial surface, thereby increasing permeability to larger molecules. Four strains (TA98, TA100, TA1535, and TA1537) also have a mutation that is the deletion of a gene coding for the DNA excision repair system (*uvrB*), which increases the sensitivity of the bacteria to mutagens. Three strains (TA98, TA100, and TA102), which contain the R-factor plasmid (pKM101) are ampicillin resistant. In addition, TA102 also contains the *hisG428* mutation on the multi-copy plasmid (pAQ1), which carries a tetracycline resistant gene. All five strains have a histidine requirement that allows for the detection of revertants on minimal medium plates. The summary of mutations of tester strains is presented in the table below.

Strain	Additional Mutations				Mutation-type Detection
	Histidine Mutation	LPS	Repair	R-factor	
TA98	*HisD3052*	Rfa	*uvrB*	pKM 101	Frameshift
TA100	*HisG46*	Rfa	*uvrB*	pKM 101	Base-pair substitution
TA102	*HisG428*	Rfa	Wild Type	pKM 101	Ochre
TA1535	*HisG46*	Rfa	*uvrB*	-	Base-pair substitution
TA1537	*HisC3076*	Rfa	*uvrB*	-	Frameshift

The bacteria will be maintained as stock cultures in liquid nitrogen, and harvested in nutrient broth with DMSO. One day before the assay, each tester strain bacteria will be cultivated, in duplicate, in 20 ml of nutrient broth with approximately 1.0 ml of the thawed stock culture. The bacteria will be incubated for 15 ± 2 hours (or until cell density reaches 1.0 x 10^9 cfu/ml), at 37 ± 1°C on an orbital shaker at a speed of 100 to 120 rpm to obtain logarithmic/early stationary growth phase cultures. If necessary, the overnight cultures will be diluted in fresh

nutrient broth, and the optical density (O.D.) will be determined at 650 nm. After obtaining the O.D., ten-fold serial dilutions will be made and the bacteria of each strain will be grown on brain heart infusion agar (BHIA) plates. After an 18 to 24 hour incubation period at 37 ± 1°C, the colonies on BHIA plates will be counted. The numbers of viable cells are usually found to give rise to a minimum of 1.0×10^8 cfu/ml.

To evaluate the potential mutagenicity of CuATSM/H_2ATSM, five dose levels of 10, 25, 30, 40, and 100 μg/plate with and without S9 will be tested for ability to induce reverse mutations. These concentrations will be adjusted depending upon the cytotoxicity and solubility of test article.

On the day of the assay, 100 μl of the test article (or positive or solvent/vehicle control article), 100 μl bacterial culture, and 500 μl S9 mixture (or sodium phosphate buffer pH 7.4 for plates without S9), will be added to 2 ml of top agar. The components will be vortexed for about 3 seconds at low to mid speed, and poured and spread evenly on minimal glucose agar plates. After the top agar solidifies, the plates will be inverted and incubated at 37 ± 1°C for 48 to 72 hours. The number of revertant colonies in each plate will be counted with an AccuCount 1000 automatic colony counter (Biologics, Inc., Gainesville, VA). For each bacterial strain and activation condition (± S9), there will be three plates for each of the solvent/vehicle control, positive control, and test article.

Each plate will be identified with a label indicating the project number, study number, description and dose of the test article (or positive or solvent/vehicle control), metabolic activation system, individual plate number, tester strain and the assay date.

In addition to positive and solvent/vehicle controls, sterility controls will be run simultaneously with the test article. Sterility control plates are of two types. The type I plates are intended to test for microbial contaminants in the test article. The test will consist of a single plate containing the test article, the overlay top agar, and the Vogel-Bonner minimal glucose medium. The type II

plates will test for microbial contaminants in the S9 mixture or sodium phosphate buffer. It consists of triplicate plates of overlay top agar, S9 mixture (or sodium phosphate buffer), and the Vogel-Bonner minimal glucose medium.

To ensure the validity of the mutagenicity data, the following criteria will be verified in the assay.

(1) Spontaneous revertants for each strain (i.e., from solvent/vehicle control)
(2) Response of strain specific positive control mutagens
(3) Viable number of bacteria, a minimum of $1x10^7$ cfu/plate
(4) Absence of toxicity at a minimum of three nontoxic dose levels.

c. Data Interpretation and Results:

Data will be presented as the number of revertants/plate along with mean and standard deviation (SD). The plate count raw data will be expressed as number of revertants/plate per concentration of test article. The data on the revertants/plate per concentration of test article will be plotted using Sigma Plot (Systat Software, Inc., Point Richmond, CA).

A test article will be concluded to be positive (or mutagenic), if the mean number of revertants in any of the strains, used at any test concentration, demonstrated at least a 2-fold increase over the mean number of revertants in the concurrent solvent/vehicle control. And, if there is a concentration dependent increase in the number of revertants per plate in that same strain. The test article will be classified as negative (or non-mutagenic), if all positive classification criteria for either strain are not met.

III. QUALITY ASSURANCE:

A. Type of Study:

This is a nonclinical laboratory study and will require compliance with the FDA Good Laboratory Practice Regulations. Data from this study will be included as part of a final report to be submitted to the FDA.

B. Standard Operating Procedures:

All operations pertaining to this study, unless specifically defined in this protocol, will be performed according to standard operating procedures of the laboratory and any deviations will be documented.

C. Protocol Amendments:

All changes in or revisions of an approved protocol and the reasons therefore will be documented, signed and dated by the Principal Investigator, Study Director and the NCI Project Officer. Amendments will be maintained with the protocol. Verbal approval for a protocol change may be granted by the NCI Project Officer, but a written amendment will follow.

D. Records:

Data and report will be audited by the IITRI Quality Assurance Unit. All raw data, and a copy of the final report will be archived in the IITRI archives for a period of one year from the date of completion of the study. At that time, the Sponsor will be contacted in order to determine the final disposition of the archival materials. IITRI's Quality Assurance Unit will maintain a complete record of the disposition of all archival materials.

IV. REPORTING AND DISCUSSION OF DATA:

A. Progress Reports:

Status reports summarizing the progress of the study will be provided at monthly intervals. These unaudited reports will detail the status of the study on the reporting date, any problems encountered, and proposed means of resolution.

B. Final Report:

The data and results of this study will be submitted as a draft report due 60 working days after completion of the experimental phase of the study is completed. The final report will be due 30 working days after the return of the draft report containing the Sponsor's comments.

This report will accurately and completely describe the study design, procedures and findings, present an analysis and summary of the data followed by conclusions derived from the analyses. The report will also include, but not necessarily be limited to: (a) a cover page which will include title, contract number, authors, laboratory address, dates of initiation and completion, and Sponsor; (b) an abstract to be placed at the beginning of the final report; (c) a comprehensive summary to be placed after the abstract; (d) the signature of the Study Director and any others deemed necessary; (e) a table of contents; (f) a statement prepared and signed by the Quality Assurance Unit which will refer to all phases of the study that were inspected; and (g) a statement of where the raw data records, reports and samples are stored.

V. REFERENCES:

FDA Redbook 2000, Toxicological principles for the safety of food ingredients: IV.C.1.a. Bacterial reverse mutation test, July 7, 2000.

Maron, D.M. and Ames, B.N. 1983. Revised methods for the *Salmonella* mutagenicity test. Mutation Research. 113:173-215.

Protocol Approvals:

Study Director: _____ 6/3/04 (Date)

Principal Investigator: _____ 6/3/04 (Date)

Project Officer: _____ 6/7/04 (Date)

Appendix B. Individual Tester Strain Data

EVALUATION OF POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM IN *SALMONELLA* REVERSE MUTATION ASSAY

Appendix B Table B-1

Mutagenicity of CuATSM/H_2ATSM in *Salmonella* Tester Strain TA98 with and without S9 – Initial Assay

TESTER STRAIN	DOSE (µg/plate)	S9	MUTAGENICITY (revertants/plate) PLATE 1	PLATE 2	PLATE 3	MEAN	SD	RSD (%)
TA98	0	+	21	32	20	24	6.7	27.7
	10	+	24	29	26	26	2.5	9.7
	25	+	30	25	36	30	5.5	18.4
	30	+	24	23	33	27	5.5	20.4
	40	+	24	30	39	31	7.5	24.4
	100	+	30	23	42	32	9.6	30.0
TA98	0	-	15	23	18	19	4.0	21.3
	10	-	18	16	9	14	4.7	33.8
	25	-	18	15	20	18	2.5	14.0
	30	-	28	18	14	20	7.2	36.1
	40	-	18	23	16	19	3.6	19.0
	100	-	24	19	22	22	2.5	11.4

DATE OF ASSAY: 07-02-2004 (Initial)

EVALUATION OF POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM IN *SALMONELLA* REVERSE MUTATION ASSAY

Appendix B Table B-2

Mutagenicity of CuATSM/H_2ATSM in *Salmonella* Tester Strain TA98
with and without S9 – Confirmatory Assay

TESTER STRAIN	DOSE (μg/plate)	S9	MUTAGENICITY (revertants/plate) PLATE 1	PLATE 2	PLATE 3	MEAN	SD	RSD (%)
TA98	0	+	34	25	24	28	5.5	19.7
	10	+	28	31	22	27	4.6	17.0
	40	+	19	33	22	25	7.4	29.5
	100	+	21	26	38	28	8.7	31.2
	500	+	34	32	23	30	5.9	19.5
	1000[a]	+	23	35	32	30	6.2	20.8
TA98	0	-	21	16	15	17	3.2	18.9
	10	-	19	22	20	20	1.5	7.6
	40	-	11	20	18	16	4.7	29.5
	100	-	10	16	13	13	3.0	23.1
	500[a]	-	23	22	32	26	5.5	21.2
	1000	-	20	24	26	23	3.1	13.3

DATE OF ASSAY: 07-09-2004 (Confirmatory)
[a]Microcolonies observed on all three plates at this dose level

EVALUATION OF POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM IN *SALMONELLA* REVERSE MUTATION ASSAY

Appendix B Table B-3

Mutagenicity of CuATSM/H_2ATSM in *Salmonella* Tester Strain TA100
with and without S9 – Initial Assay

TESTER STRAIN	DOSE (μg/plate)	S9	MUTAGENICITY (revertants/plate) PLATE 1	PLATE 2	PLATE 3	MEAN	SD	RSD (%)
TA100	0	+	114	112	122	116	5.3	4.6
	10	+	126	113	135	125	11.1	8.8
	25	+	104	128	102	111	14.5	13.0
	30	+	127	112	120	120	7.5	6.3
	40	+	133	142	127	134	7.5	5.6
	100	+	135	122	129	129	6.5	5.0
TA100	0	-	114	109	111	111	2.5	2.3
	10	-	103	117	120	113	9.1	8.0
	25	-	111	120	106	112	7.1	6.3
	30	-	98	112	104	105	7.0	6.7
	40	-	122	110	121	118	6.7	5.6
	100	-	112	118	121	117	4.6	3.9

DATE OF ASSAY: 07-02-2004 (Initial)

EVALUATION OF POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM IN *SALMONELLA* REVERSE MUTATION ASSAY

Appendix B Table B-4

Mutagenicity of CuATSM/H_2ATSM in *Salmonella* Tester Strain TA100
with and without S9 – Confirmatory Assay

TESTER STRAIN	DOSE (μg/plate)	S9	MUTAGENICITY (revertants/plate) PLATE 1	PLATE 2	PLATE 3	MEAN	SD	RSD (%)
TA100	0	+	128	135	103	122	16.8	13.8
	10	+	175	134	138	149	22.6	15.2
	40	+	128	138	113	126	12.6	10.0
	100	+	102	117	145	121	21.8	18.0
	500	+	135	126	148	136	11.1	8.1
	1000	+	127	115	132	125	8.7	7.0
TA100	0	-	108	92	106	102	8.7	8.5
	10	-	117	107	118	114	6.1	5.3
	40	-	113	101	104	106	6.2	5.9
	100	-	114	127	83	108	22.6	20.9
	500	-	131	139	158	143	13.9	9.7
	1000	-	157	253	284	231	66.2	28.7

DATE OF ASSAY: 07-09-2004 (Confirmatory)

EVALUATION OF POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM IN *SALMONELLA* REVERSE MUTATION ASSAY

Appendix B Table B-5

Mutagenicity of CuATSM/H_2ATSM in *Salmonella* Tester Strain TA102
with and without S9 – Initial Assay

TESTER STRAIN	DOSE (µg/plate)	S9	MUTAGENICITY (revertants/plate) PLATE			MEAN	SD	RSD (%)
			1	2	3			
TA102	0	+	274	247	212	244	31.1	12.7
	10	+	220	300	291	270	43.8	16.2
	25	+	283	250	296	276	23.7	8.6
	30	+	274	261	220	252	28.2	11.2
	40	+	281	262	307	283	22.6	8.0
	100	+	310	262	254	275	30.3	11.0
TA102	0	-	207	188	211	202	12.3	6.1
	10	-	149	174	197	173	24.0	13.9
	25	-	192	196	197	195	2.6	1.4
	30	-	229	228	228	228	0.6	0.3
	40	-	222	229	219	223	5.1	2.3
	100	-	209	242	226	226	16.5	7.3

DATE OF ASSAY: 07-02-2004 (Initial)

EVALUATION OF POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM IN *SALMONELLA* REVERSE MUTATION ASSAY

Appendix B Table B-6

Mutagenicity of CuATSM/H_2ATSM in *Salmonella* Tester Strain TA102
with and without S9 – Confirmatory Assay

TESTER STRAIN	DOSE (μg/plate)	S9	MUTAGENICITY (revertants/plate) PLATE 1	PLATE 2	PLATE 3	MEAN	SD	RSD (%)
TA102	0	+	351	362	314	342	25.1	7.4
	10	+	317	319	252	296	38.1	12.9
	40	+	279	297	217	264	42.0	15.9
	100	+	239	229	301	256	39.0	15.2
	500	+	320	346	301	322	22.6	7.0
	1000	+	347	334	354	345	10.1	2.9
TA102	0	-	247	244	233	241	7.4	3.1
	10	-	176	248	241	222	39.7	17.9
	40	-	217	246	273	245	28.0	11.4
	100	-	235	260	260	252	14.4	5.7
	500	-	286	253	262	267	17.1	6.4
	1000	-	450	363	373	395	47.6	12.1

DATE OF ASSAY: 07-09-2004 (Confirmatory)

EVALUATION OF POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM IN *SALMONELLA* REVERSE MUTATION ASSAY

Appendix B Table B-7

Mutagenicity of CuATSM/H_2ATSM in *Salmonella* Tester Strain TA1535
with and without S9 – Initial Assay

TESTER STRAIN	DOSE (µg/plate)	S9	MUTAGENICITY (revertants/plate) PLATE			MEAN	SD	RSD (%)
			1	2	3			
TA1535	0	+	8	7	11	9	2.1	23.1
	10	+	9	8	6	8	1.5	19.1
	25	+	13	12	10	12	1.5	12.7
	30	+	16	9	7	11	4.7	43.0
	40	+	9	14	7	10	3.6	36.1
	100	+	6	7	11	8	2.6	33.1
TA1535	0	-	7	5	7	6	1.2	19.2
	10	-	16	9	16	14	4.0	28.9
	25	-	8	12	11	10	2.1	20.8
	30	-	6	12	10	9	3.1	33.9
	40	-	6	11	13	10	3.6	36.1
	100	-	14	17	9	13	4.0	31.1

DATE OF ASSAY: 07-02-2004 (Initial)

EVALUATION OF POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM IN *SALMONELLA* REVERSE MUTATION ASSAY

Appendix B Table B-8

Mutagenicity of CuATSM/H_2ATSM in *Salmonella* Tester Strain TA1535
with and without S9 – Confirmatory Assay

TESTER STRAIN	DOSE (μg/plate)	S9	MUTAGENICITY (revertants/plate) PLATE			MEAN	SD	RSD (%)
			1	2	3			
TA1535	0	+	9	9	11	10	1.2	11.5
	10	+	9	10	6	8	2.1	26.0
	40	+	12	9	14	12	2.5	21.0
	100	+	9	10	10	10	0.6	5.8
	500	+	10	10	23	14	7.5	53.6
	1000	+	16	12	19	16	3.5	21.9
TA1535	0	-	13	16	14	14	1.5	10.9
	10	-	20[a]	19	9	16	6.1	38.0
	40	-	9	8	12	10	2.1	20.8
	100	-	5	5	9	6	2.3	38.5
	500	-	21	26	15	21	5.5	26.2
	1000[b]	-	15	13	15	14	1.2	8.2

DATE OF ASSAY: 07-09-2004 (Confirmatory)

[a]Microcolonies observed on the plate

[b]Microcolonies observed on all three plates at this dose level

EVALUATION OF POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM IN *SALMONELLA* REVERSE MUTATION ASSAY

Appendix B Table B-9

Mutagenicity of CuATSM/H_2ATSM in *Salmonella* Tester Strain TA1537
with and without S9 – Initial Assay

TESTER STRAIN	DOSE (µg/plate)	S9	MUTAGENICITY (revertants/plate) PLATE			MEAN	SD	RSD (%)
			1	2	3			
TA1537	0	+	1	1	4	2	1.7	86.6
	10	+	7	2	2	4	2.9	72.2
	25	+	3	3	6	4	1.7	43.3
	30	+	6	4	3	4	1.5	38.2
	40	+	3	5	2	3	1.5	50.9
	100	+	2	3	3	3	0.6	19.2
TA1537	0	-	5	5	5	5	0.0	0.0
	10	-	1	2	4	2	1.5	76.4
	25	-	2	3	4	3	1.0	33.3
	30	-	5	1	8	5	3.5	70.2
	40	-	5	3	5	4	1.2	28.9
	100	-	8	2	5	5	3.0	60.0

DATE OF ASSAY: 07-02-2004 (Initial)

EVALUATION OF POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM IN *SALMONELLA* REVERSE MUTATION ASSAY

Appendix B Table B-10

Mutagenicity of CuATSM/H_2ATSM in *Salmonella* Tester Strain TA1537
with and without S9 – Confirmatory Assay

TESTER STRAIN	DOSE (µg/plate)	S9	MUTAGENICITY (revertants/plate) PLATE			MEAN	SD	RSD (%)
			1	2	3			
TA1537	0	+	4	4	6	5	1.2	23.1
	10	+	5	8	5	6	1.7	28.9
	40	+	0	1	5	2	2.6	132.3
	100	+	3	3	8	5	2.9	57.7
	500	+	5	0	0	2	2.9	144.3
	1000	+	8	3	3	5	2.9	57.7
TA1537	0	-	6	1	4	4	2.5	62.9
	10	-	3	3	7	4	2.3	57.7
	40	-	7	3	2	4	2.6	66.1
	100	-	3	0	3	2	1.7	86.6
	500	-	5	2	1	3	2.1	69.4
	1000	-	1	5[a]	5[a]	4	2.3	57.7

DATE OF ASSAY: 07-09-2004 (Confirmatory)

[a]Microcolonies presented on the plate, and included in plate counts

EVALUATION OF POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM IN *SALMONELLA* REVERSE MUTATION ASSAY

Appendix B Table B-11

Negative (Spontaneous Reversions) and Positive Control Assays of *Salmonella typhimurium* Tester Strains – Initial Assay

STRAIN	CONTROL SUBSTANCE	CONCENTRATION	S9 ACTIVATION	MUTAGENICITY (revertant colonies/plate) PLATE 1	PLATE 2	PLATE 3	MEAN	SD	RSD (%)
TA98	DMSO	0.1 ml/plate	+	21	32	20	24	6.7	27.7
	2-AA	2 µg/plate	+	552	511	569	544	29.8	5.5
	2-AF	20 µg/plate	+	1494	1332	1710	1512	189.6	12.5
TA98	DMSO	0.1 ml/plate	-	15	23	18	19	4.0	21.3
	Daunomycin	10 µg/plate	-	160	178	173	170	9.3	5.5
TA100	DMSO	0.1 ml/plate	+	114	112	122	116	5.3	4.6
	2-AA	10 µg/plate	+	1442	1477	1458	1459	17.5	1.2
	2-AF	20 µg/plate	+	1075	979	972	1009	57.6	5.7
TA100	DMSO	0.1 ml/plate	-	114	109	111	111	2.5	2.3
	MMS	5 mg/plate	-	1533	1437	1473	1481	48.5	3.3
TA102	DMSO	0.1 ml/plate	+	274	247	212	244	31.1	12.7
	Danthron	30 µg/plate	+	554	711	717	661	92.4	14.0
TA102	DMSO	0.1 ml/plate	-	207	188	211	202	12.3	6.1
	Cumene H.	100 µg/plate	-	696	754	702	717	31.9	4.4
TA1535	DMSO	0.1 ml/plate	+	8	7	11	9	2.1	23.1
	2-AA	2 µg/plate	+	130	124	100	118	15.9	13.5
TA1535	DMSO	0.1 ml/plate	-	7	5	7	6	1.2	19.2
	NaN_3	10 µg/plate	-	880	901	942	908	31.5	3.5
TA1537	DMSO	0.1 ml/plate	+	1	1	4	2	1.7	86.6
	2-AA	10 µg/plate	+	76	75	82	78	3.8	4.9
TA1537	DMSO	0.1 ml/plate	-	5	5	5	5	0.0	0.0
	ICR-191	10 µg/plate	-	1741	1481	1481	1568	150.1	9.6

DATE OF ASSAY: 07-02-2004 (Initial)

EVALUATION OF POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM IN *SALMONELLA* REVERSE MUTATION ASSAY

Appendix B Table B-12

Negative (Spontaneous Reversions) and Positive Control Assays of *Salmonella typhimurium* Tester Strains – Confirmatory Assay

STRAIN	CONTROL SUBSTANCE	CONCENTRATION	S9 ACTIVATION	MUTAGENICITY (revertant colonies/plate) PLATE 1	PLATE 2	PLATE 3	MEAN	SD	RSD (%)
TA98	DMSO	0.1 ml/plate	+	34	25	24	28	5.5	19.7
	2-AA	2 µg/plate	+	571	626	651	616	40.9	6.6
	2-AF	20 µg/plate	+	1891	2017	1883	1930	75.2	3.9
TA98	DMSO	0.1 ml/plate	-	21	16	15	17	3.2	18.9
	Daunomycin	10 µg/plate	-	335	333	217	295	67.6	22.9
TA100	DMSO	0.1 ml/plate	+	128	135	103	122	16.8	13.8
	2-AA	10 µg/plate	+	1374	1409	1274	1352	70.1	5.2
	2-AF	20 µg/plate	+	869	864	773	835	54.0	6.5
TA100	DMSO	0.1 ml/plate	-	108	92	106	102	8.7	8.5
	MMS	5 mg/plate	-	783	501	210	498	286.5	57.5
TA102	DMSO	0.1 ml/plate	+	351	362	314	342	25.1	7.4
	Danthron	30 µg/plate	+	709	684	494	629	117.6	18.7
TA102	DMSO	0.1 ml/plate	-	247	244	233	241	7.4	3.1
	Cumene H.	100 µg/plate	-	1010	910	740	887	136.5	15.4
TA1535	DMSO	0.1 ml/plate	+	9	9	11	10	1.2	11.5
	2-AA	2 µg/plate	+	129	142	160	144	15.6	10.8
TA1535	DMSO	0.1 ml/plate	-	13	16	14	14	1.5	10.9
	NaN_3	10 µg/plate	-	921	987	1029	979	54.4	5.6
TA1537	DMSO	0.1 ml/plate	+	4	4	6	5	1.2	23.1
	2-AA	10 µg/plate	+	136	109	78	108	29.0	26.9
TA1537	DMSO	0.1 ml/plate	-	6	1	4	4	2.5	62.9
	ICR-191	10 µg/plate	-	1510	1772	1691	1658	134.1	8.1

DATE OF ASSAY: 07-09-2004 (Confirmatory)

Appendix C. Confirmatory Assays of Tester Strains

EVALUATION OF POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM IN *SALMONELLA* REVERSE MUTATION ASSAY

Appendix C Table C-1

Phenotypic and Genotypic Characterization of *Salmonella typhimurium* Tester Strains

	GROWTH		SENSITIVITY			
Tester Strains	**without Histidine**	**with Histidine**	**rfa to Crystal Violet**	***uvrB* to Ultraviolet Light**	**pKM101 to Ampicillin**	**pAQ1 to Tetracycline**
TA98	No Growth	Growth	Sensitive	Sensitive	Resistant	Sensitive
TA100	No Growth	Growth	Sensitive	Sensitive	Resistant	Sensitive
TA102	No Growth	Growth	Sensitive	Resistant	Resistant	Resistant
TA1535	No Growth	Growth	Sensitive	Sensitive	Sensitive	Sensitive
TA1537	No Growth	Growth	Sensitive	Sensitive	Sensitive	Sensitive

Phenotypic/genotypic confirmation is performed on a quarterly basis.
This confirmation was performed on July 19 - 22, 2004.

EVALUATION OF POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM IN *SALMONELLA* REVERSE MUTATION ASSAY

Appendix C Table C-2

Viability of *Salmonella typhimurium* Tester Strain Cells Used in the Mutagenicity Assay

Initial Assay

Dilution Factor	Tester Strain	Plate Count (cfu/mL) 1	2	3	MEAN	SD	%RSD	cfu/mL
10^7	TA98	52	46	43	47	4.6	9.8	4.7×10^8
10^7	TA100	34	39	29	34	5.0	14.7	3.4×10^8
10^7	TA102	123	137	110	123	13.5	11.0	1.2×10^9
10^7	TA1535	64	52	58	58	6.0	10.3	5.8×10^8
10^6	TA1537	263	254	219	245	23.2	9.5	2.5×10^8

Confirmatory Assay

Dilution Factor	Tester Strain	Plate Count (cfu/mL) 1	2	3	MEAN	SD	%RSD	cfu/mL
10^7	TA98	79	90	78	82	6.7	8.1	8.2×10^8
10^7	TA100	48	51	54	51	3.0	5.9	5.1×10^8
10^7	TA102	145	172	147	155	15.0	9.7	1.6×10^9
10^7	TA1535	66	81	67	71	8.4	11.8	7.1×10^8
10^7	TA1537	110	84	88	94	14.0	14.9	9.4×10^8

Date of Assay: July 2, 2004 (Initial), July 9, 2004 (Confirmatory)

EVALUATION OF POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM IN *SALMONELLA* REVERSE MUTATION ASSAY

Appendix C Table C-3

Historical Data of Positive and Vehicle/Solvent Control Response

Tester Strain	Control Article	Concentration	S9	Mutagenicity (revertants/plate) Historical[a] Mean	SD	Minimum	Maximum	Number of Plates
TA98	Vehicle	0.1 ml/plate	+	32	9.1	15	51	80
	2-AA	2 µg/plate	+	704	208.1	233	1082	81
	2-AF	20 µg/plate	+	1620	275.4	1242	2017	80
	Vehicle	0.1 ml/plate	-	23	6.5	9	38	81
	Daunomycin	10 µg/plate	-	294	162.9	104	782	81
TA100	Vehicle	0.1 ml/plate	+	104	43.3	57	263	81
	2-AA	10 µg/plate	+	1452	381.3	817	1989	78
	2-AF	20 µg/plate	+	1076	302.2	582	1600	78
	Vehicle	0.1 ml/plate	-	106	33.7	62	206	81
	MMS	5 mg/plate	-	1229	326.7	501	1762	78
TA102	Vehicle	0.1 ml/plate	+	312	37.4	212	387	81
	Danthron	30 µg/plate	+	674	101.1	494	870	80
	Vehicle	0.1 ml/plate	-	220	41.0	126	328	81
	Cumene H.	100 µg/plate	-	906	237.1	560	1242	78
TA1535	Vehicle	0.1 ml/plate	+	10	4.4	4	28	81
	2-AA	2 µg/plate	+	180	63.8	57	366	80
	Vehicle	0.1 ml/plate	-	11	4.8	3	26	81
	NaN_3	10 µg/plate	-	884	186.3	590	1189	79
TA1537	Vehicle	0.1 ml/plate	+	5	3.4	1	19	80
	2-AA	10 µg/plate	+	69	49.0	9	196	81
	Vehicle	0.1 ml/plate	-	4	2.7	1	14	77
	ICR-191	10 µg/plate	-	1006	380.0	298	1804	79

[a]IITRI's positive and vehicle/solvent controls historical data were based on individual plate counts; Assay dates from February 13 to July 9, 2004.

Appendix D. Test and Control Article Certificates of Analysis

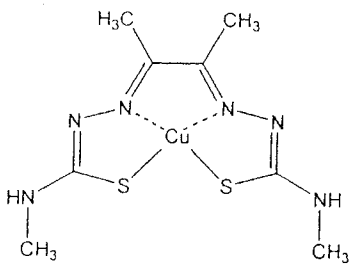
Appendix D - Test Article Certificate of Analysis

6336-151
6336-152

COPY

2073-002-002
2073-002-003
2073-002-001-002

Cu-ATSM



Elemental Analysis;

	C	H	N	S
Expected; Calc for $CuC_8H_{14}S_2N_6$	29.85	4.38	26.11	19.92
Found (Cu JSL(1))	29.83	4.54	25.92	20.04
Found (Cu JSL(2))	29.54	4.50	25.66	20.00
Found (Cu JSL(3))	29.74	4.55	25.95	19.86

Mass Spectra;

LRFAB: Peak match to $[M + H]^+$ m/z = 321.9855

HRFAB: Peak match to $[M + H]^+$ m/z = 322.0095

ESI +ve: A 1 mg/ml solution of Cu-ATSM (M) was made up by dissolving 1 mg of material into 1 mL of ethanol. Of this freshly prepared solution 20 µL was removed and added to 200 µL of a 1:1 mixture of water and methanol. This was then directly infused at a flow rate of 10 µL/min into the water ZQ 4000 mass spectrometer. The conditions of the mass spectrometer were then adjusted to generate a signal of maximum intensity. Data was then collected using an ESI probe operating in the positive mode for a time frame of 2 minutes over the M/Z range of 150-600 Da. The major peak was observed at 321.88 Da which corresponds to $C_8H_{15}N_6S_2Cu$ or $[M + H]^+$. The isotopic distribution pattern around this peak matched exactly the theoretical pattern generated by Cu-ATSM.

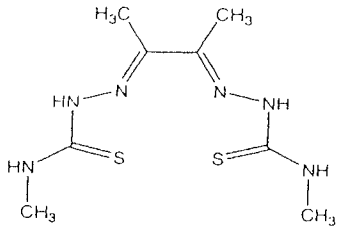
Appendix D - Test Article Certificate of Analysis (cont.)

COPY

6336-151
6336-152

2073-002-002
2073-002-003
2073-002-001-002

H_2ATSM



Elemental Analysis;

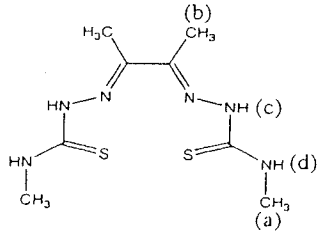
	C	H	N
Expected; Calc for $C_8H_{16}S_2N_6.¼H_2O$	36.40	6.30	31.83
Found (JSL 3697)	36.48	6.23	31.33
Found (JSL 3697 (b))	36.59	6.15	31.77

I have a large number of elemental analysis results, shown above are two representative samples. The results from an identical sample run twice do vary. This leads to the assumption that water is present. When calculated for different amounts of water present the elemental analysis result for all samples are consistent. The presence of water is confirmed by the NMR spectra (see below).

Mass Spectra;
HRFAB: Peak match to $[M + H]^+$ m/z = 261.0956

HREI: Molecular Ion Peak m/z = 260.0878

NMR δ(ppm), in DMSO-d_6;
The ^{1}H-NMR is somewhat difficult to interpret due to the tautomeric nature of the ligand.



2.203 (s, C<u>H</u>$_3$ (b), 6H); 3.011-3.026 (d, C<u>H</u>$_3$ (a), 6H); Doublet at 8.365 and singlet at 10.214 are not possible to assign due to tautomeric nature, however, they do correspond to the N<u>H</u> and intergrate for (C) and (d) as 4H consistent with the structure. Water is prensent in the NMR (confirming elemental analysis) at 3.338 ppm.

The ^{13}-NMR is very clean and consistent with structure. 11.657 (s, <u>C</u>H$_3$ (b)); 31.207 (s, <u>C</u>H$_3$ (a)); 147.977 (<u>C</u>=S); 178.471 (<u>C</u>=N).

COPY

TA # V108C

SIGMA-ALDRICH

Certificate of Analysis

Product Name	Dimethyl sulfoxide
Product Number	D1435
CAS Number	67-68-5
Molecular Formula	C_2H_6OS
Molecular Weight	78.13

TEST	SPECIFICATION	LOT 033K0640 RESULTS
IDENTITY	PASS	PASS
SPECIFIC GRAVITY	1.095 TO 1.101	1.099
CONGEALING TEMPERATURE	NLT 18.3 DEG C INDICATING NLT 99.9% C2H6OS	18.4 DEG C
REFRACTIVE INDEX	1.4755 TO 1.4775	1.4761
ACIDITY	PASS	PASS
WATER CONTENT BY KARL FISCHER	NMT 0.1%	0.017%
UV ABSORBANCE	PASS	PASS
SUBSTANCES DARKENED BY POTASSIUM HYDROXIDE	PASS	PASS
LIMIT OF DIMETHYL SULFONE	NMT 0.1%	PASS
LIMIT OF NONVOLATILE RESIDUE	PASS	PASS
		ALL RESULTS SUPPLIER DATA
		MEETS CURRENT USP REQUIRMENTS
SHELF LIFE SOP QC-12-006	2 YEARS	MARCH 2005
QC ACCEPTANCE DATE		MARCH 2003

Lori Schulz, Manager
Analytical Services