EVALUATION OF THE POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM (NSC-D729307) IN THE MOUSE LYMPHOMA ASSAY

FINAL REPORT

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Testing Facility:

IIT Research Institute (IITRI)
Life Sciences Group
10 West 35th Street
Chicago, IL 60616-3799

Authors:

Patrick T. Curry, Ph.D.
Study Director

David L. McCormick, Ph.D., D.A.B.T.
Principal Investigator

Sponsor:

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

NCI Project Officer:

Elizabeth Glaze, Ph.D.

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EVALUATION OF THE POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM (NSC-D729307) IN THE MOUSE LYMPHOMA ASSAY

ABSTRACT

The potential mutagenic activity of Copper-diacetyl-bis (N^4-methylthiosemicarbazone)/diacetyl-bis-(N^4-methylthiosemicarbazone) (CuATSM/H_2ATSM; NSC-D729307) was tested in the mouse lymphoma assay. The L5178Y $TK^{+/-}$ mouse lymphoma cell line (Clone 3.7.2.C) was used to test the mutagenicity of CuATSM/H_2ATSM with and without metabolic activation. Aroclor 1254-induced rat liver S9 fraction with co-factors was used as the metabolic activation system.

The solubility of CuATSM/H_2ATSM in dimethylsulfoxide (DMSO) permitted the conduct of *in vitro* studies at drug concentrations up to 100 μg/ml. In the initial mutagenicity assay, which also served as a cytotoxicity range-finding study, cytotoxicity was assessed by comparing the relative total growth (RTG) of the treated cultures to the vehicle control article cultures. CuATSM/H_2ATSM was initially tested up to 100 μg/ml in both the presence and absence of metabolic activation with a 4-hour exposure. There were no mutant colonies formed in any of the trifluorothymidine (TFT) culture plates, probably due to cytotoxicity (data not shown). However, the viable colony count plates (non-selective media, contained no TFT) did have colonies at dose levels of 25 μg/ml or less (dose levels of 25 μg/ml and greater were excessively toxic; i.e., the relative total growth (RTG) was less than 10%). From these plates a relative total growth was established for each dose level and a dose range for an ensuing repeat initial assay was selected. In the repeat initial mutagenicity assay, in both the presence and absence of metabolic activation, a top dose level of 25 μg/ml was included to confirm the excessive toxicity previously observed at this dose level. The remaining dose levels in the repeat assay were 3.0, 5.0, 7.0, 10.0, 12.0, 15.0, 18.0, and 20.0 μg/ml (the 3.0 μg/ml dose level was not needed in the +S9 portion of the assay and was therefore not analyzed). In the repeat initial mutagenicity assay in the non-activated test system dose levels of 15.0 μg/ml and greater demonstrated excessive cytotoxicity (the 20 μg/ml dose level was unanalyzable). Excessive cytotoxicity was indicated when the sample RTG was less than 10% relative to the vehicle control (cytotoxicity of 90%). The remaining dose levels ranged from 33% to 85% cytotoxicity relative to the vehicle control. In the S9-activated test system the 20.0 and 25.0 μg/ml dose levels demonstrated excessive cytotoxicity (the 25.0 μg/ml was unanalyzable). The remaining dose levels ranged from non-toxic to 71% cytotoxicity relative to the vehicle control.

In the initial range-finding assay, in both the absence and presence of metabolic activation the mutation frequency was not elevated by a factor of two or more times the vehicle mutation frequency in any of the dose levels tested.

In the definitive mutagenicity assay, in the absence of metabolic activation with a 24-hour exposure, dose levels of 0.50, 1.00, 3.00, 5.00, 10.00, 12.00 and 15.00 μg/ml were tested. Dose levels of 10.00 μg/ml or greater were not cloned due to excessive cytotoxicity. Cytotoxicity was observed during Day 1 and Day 2 cell counts (prior to cloning) as a decrease of 80% or greater in the relative suspension growth (RSG < 20%) relative to the vehicle control. The RTG in the absence of metabolic activation for the remaining dose levels (which were cloned) ranged from 50% to 12% of the vehicle control. In the presence of metabolic activation with a 4-hour exposure dose levels of 10.00, 12.00, 15.00, 18.00 and 20.00 μg/ml were tested. All tested dose levels were analyzable and the cytotoxicity ranged from non-toxic to 72% relative to the vehicle control.

In the definitive mutagenicity assay, in both the absence and presence of metabolic activation, the mutation frequency was not elevated by a factor of two or more times the vehicle mutation frequency in any of the doses tested.

CuATSM/H_2ATSM was therefore concluded to be negative for mutagenicity in the mouse lymphoma assay.

COMPREHENSIVE SUMMARY

Title: Evaluation of the Potential Mutagenic Activity of CuATSM/H_2ATSM (NSC-729307) in the Mouse Lymphoma Assay

Strain: L5178Y$^{+/-}$ (Clone 3.7.2C Cell Line)

Dose Groups: Cytotoxicity Assay (Initial Mutagenicity Assay):

Mouse lymphoma cells were treated with CuATSM/H_2ATSM for 4 hours at concentrations up to 100 µg/ml in both the absence and presence of metabolic activation. There were no mutant colonies formed in any of the trifluorothymidine (TFT) culture plates, probably due to cytotoxicity (data not shown). The viable colony count plates (non-selective media, contained no TFT) did have colonies at dose levels of 25 µg/ml or less. From these plates a relative total growth was established for each dose level (see Table 1) and used to select a dose range for the repeat initial mutagenicity assay. Dose levels of 25 µg/ml and greater were excessively toxic (relative total growth (RTG) was less than 10%). In the repeat initial assay, in both the presence and absence of metabolic activation, a top dose level of 25 µg/ml was included to confirm the excessive toxicity previously observed at this dose level. The remaining dose levels in the repeat assay were 3.0, 5.0, 7.0, 10.0, 12.0, 15.0, 18.0, and 20.0 µg/ml.

Mutagenicity Assay (Confirmatory Mutagenicity Assay):

Mouse lymphoma cells were exposed to CuATSM/H_2ATSM at concentrations of 0.50, 1.00, 3.00, 5.00, 10.00, 12.00 and 15.00 µg/ml without S9 for 24 hours and 10.00, 12.00, 15.00, 18.00 and 20.00 µg/ml with S9 for 4 hours. Methyl methanesulfonate (26.8 µg/ml) without S9 and benzo[a]pyrene (2.0 µg/ml) with S9 were used as positive controls. Dimethylsulfoxide (with and without S9) was used as the vehicle.

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

Vehicle: Dimethylsulfoxide (DMSO)

RESULTS:

Cytotoxicity Assay: In the repeat cytotoxicity assay (repeat initial mutagenicity assay) in the non-activated test system, dose levels of 15.0 μg/ml and greater demonstrated excessive cytotoxicity (the 20.0 μg/ml dose level was unanalyzable). Cytotoxicity was defined as the percentage of inhibition of cell growth relative to the vehicle control (100%-RTG). Excessive culture growth inhibition, or excessive cytotoxicity, was indicated when the sample RTG was less than 10% relative to the vehicle control (indicating cytotoxicity of 90%). The remaining dose levels, ranging from 33% to 85% cytotoxicity relative to the vehicle, are listed in Table 3. In the S9-activated test system the 20 and 25.0 μg/ml dose levels demonstrated excessive cytotoxicity (the 25.0 μg/ml dose level was unanalyzable). The remaining dose levels, which ranged from non-toxic to 71% cytotoxicity relative to the vehicle control, are listed in Table 4.

In the repeat initial mutagenicity assay, in both the absence and presence of metabolic activation, the mutation frequency was not elevated by a factor of at least two or more times the vehicle mutation frequency in any of the doses tested. The mutation frequencies are listed in Tables 3 and 4.

Mutagenicity Assay (Confirmatory Mutagenicity Assay):

In the confirmatory mutagenicity assay, in the absence of metabolic activation with a 24-hour exposure, dose levels of 10 μg/ml or greater were not analyzable due to excessive cytotoxicity. The relative total growth values in the absence of metabolic activation for the remaining dose levels ranged from 59% to 12% of the vehicle control. In the presence of metabolic activation all tested dose levels were analyzable and the relative total growth values ranged from non-toxic to 28% of the vehicle control.

In the definitive mutagenicity assay, in both the absence and presence of metabolic activation, the mutation frequency was not elevated by a factor of at least two or more times the vehicle mutation frequency in any of the

doses tested. The RTGs and the mutation frequencies are listed in Tables 5 and 6.

CONCLUSION: CuATSM/H_2ATSM was not mutagenic in the mouse lymphoma assay in the presence or absence of metabolic activation.

EVALUATION OF THE POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM (NSC-D729307) IN THE MOUSE LYMPHOMA ASSAY

Study Initiation Date: May 25, 2004
Experimental Initiation Date: June 16, 2004
Experimental Termination Date: August 11, 2004

FOREWORD

This report describes the testing of potential mutagenic activity of CuATSM/H_2ATSM in the mouse lymphoma mutation assay, a study conducted by IIT Research Institute (IITRI) for the National Cancer Institute. The NCI Project Officer for the study was Elizabeth Glaze, Ph.D.

Patrick T. Curry, Ph.D., 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, and Glenn B. Miller, M.S., C.Q.M., Manager, IITRI Quality Assurance, was responsible for the quality assurance program.

Patrick T. Curry 1/18/05

Patrick T. Curry, Ph.D. Date
Study Director
Life Sciences Group

1/18/05

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

EVALUATION OF THE POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM (NSC-D729307) IN THE MOUSE LYMPHOMA ASSAY

GLP COMPLIANCE STATEMENT

This study was conducted in compliance with the US Food and Drug Administration Good Laboratory Practice Regulations (21CFR Part 58), except that no analyses for concentration, homogeneity and stability of the prepared dosing formulations or any of the other agents used in the study were performed. The compound identity, strength, quality, stability and purity as well as documentation of methods of synthesis, fabrication or derivation were the responsibility of the Sponsor. 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.

Patrick T. Curry 1/18/05

Patrick T. Curry, Ph.D. Date
Study Director

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EVALUATION OF THE POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM (NSC-D729307) IN THE MOUSE LYMPHOMA ASSAY

I. INTRODUCTION

The mouse lymphoma assay (MLA) is a short-term assay designed to detect forward gene mutations induced by mutagens at the heterozygous thymidine kinase (TK) locus and is capable of quantifying genetic alterations such as point mutations, large scale chromosomal changes and recombination. The L5178Y $TK^{+/-}$ cell line is sensitive to the cytotoxic effects of the pyrimidine analogue, trifluororthymidine (TFT), and when treated *in vitro* with mutagenic and/or carcinogenic agents, $TK^{+/-}$ is mutated to the $TK^{-/-}$ genotype which confers TFT-resistance. The mutant cells then proliferate and form colonies when cloned in soft agar medium containing the selective agent TFT. A substantial database of mutagenic and/or carcinogenic chemicals has been established for the mouse lymphoma assay (Clive et al., 1983 and 1995; Turner et al. 1984; Moore et al., 2000). This test is suitable as a short-term mutagenicity screening assay to predict chemical carcinogenicity (FDA Redbook 2000).

The objective of this study was to determine the potential mutagenic activity of CuATSM/H_2ATSM with and without S9 in the mouse lymphoma assay.

II. MATERIALS AND METHODS

A. Cell Line and Media: The L5178Y $TK^{+/-}$ (Clone 3.7.2C) cell line, was purchased from American Type Culture Collection (ATCC, Manassas, VA), and maintained in log phase growth by serial subculturing. To reduce the frequency of spontaneous $TK^{-/-}$ mutants, cell cultures were cleansed of pre-existing $TK^{-/-}$ mutants by exposing them to thymidine, hypoxanthine, methotrexate and glutamine (THMG) for approximately 24 hours to select against the $TK^{-/-}$ phenotype.

The cells were routinely cultured in RPMI-1640 supplemented with 25mM HEPES and 2mM L-glutamine, (GIBCO BRL, Grand Island, NY), 8.2% heat-inactivated horse serum (GIBCO BRL), antibiotics (Penicillin G, 17.9 units/ml and streptomycin sulfate, 17.9 µg/ml, GIBCO BRL), sodium pyruvate (0.20 mg/ml, GIBCO BRL) and Pluronic® F-68 (8.1mg/ml, GIBCO BRL) hereafter referred to as complete media. During treatment with test compounds the media (referred to as treatment media) consisted of a 2:1:1 mixture of conditioned media (the cell-free media in which the cells have grown), complete media, and RPMI-1640. The cloning media was complete media containing 0.24% granulated

agar BBL + additional 10% HS (Becton Dickinson Company, Cockeysville, MD). For selection, the cloning media was supplemented with 1 μg/ml TFT (Sigma).

B. Test and Vehicle 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 C.

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 C.

C. Analytical Chemistry: Analyses for concentration, homogeneity and stability of the prepared dosing formulations were not performed by IITRI.

D. Experimental Design and Procedures:

1. Test Article Formulation: For the repeated initial assay (July 7, 2004), a 10,000 μg/ml or 10 mg/ml (w/v) test article stock solution was prepared on June 14, 2004 by mixing 0.050 g of CuATSM/H_2ATSM with 5 ml (*q.s.*) of dimethylsulfoxide (DMSO). This stock solution was stored frozen at approximately 4°C until the day of the assay, at which time it was then thawed and used for the dose formulation preparation. For the confirmatory assay (July 27, 2004), a 2,000 μg/ml or 2 mg/ml (w/v) test article stock solution was prepared on the day of the assay by mixing 0.010 g of CuATSM/H_2ATSM with 5 ml (*q.s.*) of dimethylsulfoxide (DMSO). This stock solution was kept at room temperature and was used on the same day for dose formulation preparation by adding appropriate volumes of the vehicle.

2. Positive Control Articles: The positive control articles were methyl methanesulfonate (MMS; Acros Organics N.V., Fairlawn, NJ) in the absence of metabolic activation (-S9) and benzo[a]pyrene (BaP; Sigma Chemical Company, St. Louis, MO) in the presence of metabolic activation (+S9). MMS is a direct acting mutagen and BaP is a promutagen that requires biotransformation with liver enzymes to elicit a mutagenic response.

3. Vehicle Control Article: DMSO alone and DMSO with S9 treated cultures were used as vehicle controls.

4. Metabolic Activation System: Aroclor 1254-induced rat liver post-mitochondrial fraction (S9) with co-factors (Molecular Toxicology Inc., Boone, NC) containing 37.2 mg/ml of protein, was used as the metabolic activation system.

5. Assay Procedure:

a. Treatment: An initial experiment was performed to determine the cytotoxic range of the test article. This experiment (the cytotoxicity assay or initial mutagenicity assay) was designed to be used as a mutagenicity assay in the event that a sufficient number of dose levels survived for analysis. Limited solubility of the test article/DMSO stock solution in culture media and the requirement to keep the final concentration of the vehicle to 1% or less restricted the top test dose to 100 µg/ml. CuATSM/H_2ATSM was initially tested up to 100 µg/ml with a 4-hour exposure in both the presence and absence of metabolic activation. There were no mutant colonies formed in any of the trifluorothymidine (TFT) culture plates, probably due to cytotoxicity (data not shown). However, the viable colony count plates (non-selective media, contained no TFT) did have colonies at dose levels of 25 µg/ml or less (dose levels of 25 µg/ml and greater were excessively toxic; i.e., the relative total growth (RTG) was less than 10%). From these plates a relative total growth was established for each dose level and a dose range for an ensuing repeat initial assay was selected. In a repeat assay, the test system was exposed to the test article for 4 hours in both the presence and absence of metabolic activation. The test article dose levels used for the repeated cytotoxicity assay (or initial mutagenicity assay) are presented in the table below.

Treatment Groups in the Cytotoxicity Assay	S9
Media Control	0
Media Control	5%
Vehicle Control Article, 1.0%	0
Vehicle Control Article, 1.0%	5%
Methyl Methanesulfonate –26.8 µg/ml	0
Benzo[a]pyrene (BaP) – 2.0µg/ml	1%
CuATSM/H_2ATSM – 3.00, 5.00, 7.00, 10.00, 12.00, 15.00, 18.00, 20.00 and 25.00 µg/ml	0
CuATSM/H_2ATSM – 5.00, 7.00, 10.00, 12.00, 15.00, 18.00, 20.00 and 25.00 µg/ml	5%

Treatment with CuATSM/H_2ATSM in the initial cytotoxicity experiment resulted in a sufficient number of treatment groups demonstrating adequate cytotoxicity for cloning and mutation analysis. Based on the cytotoxicity results from the initial experiment, dose levels for the confirmatory mutagenicity assay were adjusted as shown in the table below. The exposure period in the presence of metabolic activation was 4 hours. In the absence of metabolic activation the exposure period was 24 hours.

Treatment Groups in the Definitive Mutagenicity Assay	**S9**
Media Control	0
Media Control	5%
Vehicle Control Article, 1.0%	0
Vehicle Control Article, 1.0%	5%
Methyl Methanesulfonate – 26.8 µg/ml	0
Benzo[a]pyrene (BaP) – 2.0 µg/ml	5%
CuATSM/H_2ATSM – 0.50, 1.00, 3.00, 5.00, 10.00, 12.00 and 15.00 µg/ml	0
CuATSM/H_2ATSM – 10.00, 12.00, 15.00, 18.00 and 20.00 µg/ml	5%

b. Exposure Period: On Day 0, L5178Y $TK^{+/-}$ cells growing in logarithmic phase were treated in individual 50 ml culture tubes. Each tube consisted of 7.9 ml of cell suspension ($7.6x10^5$ cells/ml) in treatment media. A 2.0 ml addition of S9 mixture or media, and 0.1 ml of RPMI, test article or control article (positive or vehicle) reduced the cell concentration to 6.0×10^5 cells/ml. Two cultures were treated at each dose level. Following addition of the test article, the tubes were gassed with a 5% CO_2 and air mixture, and then placed in a roller drum (40-45 RPM) at 37°C for the exposure period.

c. Incubation Period: At the end of the exposure period, cells were pelleted, washed with media, and then re-suspended in 20 ml of complete media at a cell concentration of 2.0×10^5 cells/ml. Tubes were gassed and transferred to the roller drum for 20-24 hours.

d. Expression Period: On Day 1, approximately 20-24 hours after treatment, the cultures were counted and diluted with fresh complete media to a concentration of $2.0x10^5$ cells/ml, gassed with a 5% CO_2 and air mixture, and returned to the roller drum. On Day 2 (approximately 40-48 hours after the Exposure Period) the

cells were re-suspended at $3.0x10^5$cells/ml, and the relative suspension growth and cumulative suspension growth were established for each concentration of CuATSM/H_2ATSM. Samples with a two-day cumulative relative suspension growth less than 10% were not cloned.

e. Cloning: Cloning media was prepared and dispensed into flasks prior to cloning. The TFT flasks (A) and viable count flasks (C) had 100 ml of cloning media dispensed into them, and the dilution flasks (B) had 50 ml dispensed into them. Approximately 30 minutes after the cell cultures were re-suspended (Day 2), the samples were centrifuged at 220 x g for 5 minutes, and all but 2-3 ml of the supernatant was removed. The culture was mixed with a pasteur pipette and transferred to the TFT flask A. After 30-45 minutes, 1.0 ml of cell suspension was transferred from the TFT flask A to the dilution flask B. A 1.0 ml aliquot of TFT (1 μg/ml) was added to the TFT flask A to select for L5178Y $TK^{-/-}$ cells. Both flasks (A and B) were then placed in a shaker incubator. The TFT flask A was in the shaker incubator for at least 15 minutes, and dilution Flask B was in for 30-45 minutes. Dilution flask B was removed from the shaker incubator and 1.0 ml of cell suspension from flask B was transferred to the viable count flask C. Dilution flask B was then discarded and viable count flask C was placed in the shaker incubator for at least 15 minutes. The contents of TFT flask A and viable count flask C were pipetted into 3 separate (100 mm x 20 mm) petri plates to yield approximately 33 ml of cloning media cell suspension per dish. The dishes were allowed to cool for 10-15 minutes at 0-6°C to expedite gelling. The plates were stacked and placed in an incubator at 37°C, 5% CO_2 and air, and 95% relative humidity for 12-15 days.

f. Culture Period: Approximately 1.0×10^6 cells per mutant plate and 200 cells per viable count plate were seeded in the respective plates. After the 12-15 day culture period both large and small mutant colonies in the plates were counted using a BIOTRAN II automatic colony counter (New Brunswick Scientific, Edison, NJ).

g. Presentation of Data:

i. Positive control article mutant frequencies with and without S9 (benzo[a]pyrene or methyl methanesulfonate respectively) used to demonstrate mutant recovery and detectibility (see Tables 3-6).

ii. Day one cell concentration and relative suspension growth (RSG, Tables B-2, B-4, B-6, B-11, B-16 and B-21), an indicator of short term cytotoxicity.

iii. Day two cell concentration, RSG and cumulative RSG (B-2, B-4, B-6, B-11, B-16 and B-21), an indicator of short term cytotoxicity.

iv. Relative total growth (RTG); an indicator of relative cell survival (Tables 1-6).

v. Number of mutant colonies including unsized, small and large colonies (Tables B-7 through B-9, B-12 through B-14, B-17 through B-19, and B-22 through B-24).

vi. Mean absolute cloning efficiency (Tables B-1, B-3, B-5, B-10, B-15 and B-20). The mean absolute cloning efficiency of the vehicle control should be at least 60%.

vii. Results of test article (CuATSM/H_2ATSM), vehicle and positive (MMS and B[a]P) control articles: RSG, RTG and mutant frequencies (calculated as number of mutant colonies per 10^6 viable cells) with and without S9 as mean ± standard deviation, Tables 3-6; and Tables B-1 - B-24).

viii. The following equations were utilized to calculate the data.

Relative Suspension Growth (RSG):

$$\frac{\textit{Cell concentration of individual culture}}{\textit{Average concentration of vehicle culture}} \quad X\,100$$

Cumulative RSG:

$$\frac{\textit{Day 1 RSG X Day 2 RSG}}{100}$$

Cloning Efficiency (CE):

$$\frac{\textit{Average no. of colonies in viable count (VC) plates}}{\textit{Total no. of cells seeded per VC plate}} \; X\,100$$

Relative Cloning Efficiency (RCE):

$$\frac{\textit{Average no. of colonies in test VC plates}}{\textit{Average no. of colonies in vehicle control VC plates}} \; X\,100$$

Relative Total Growth (RTG):

$$\frac{\textit{Cumulative RSG X RCE}}{100}$$

Mutation Frequency (MF):

$$\frac{\textit{Average no. of colonies per TFT plate}}{\textit{Cloning efficiency (CE)}} \; X\,100$$

ix. The criteria for a valid assay were: (1) the positive controls exhibit a mutant frequency at least twice that of the vehicle control with an RTG of 10% or greater, (2) the cloning efficiency of the vehicle control is 60% or greater, and (3) the mutant frequencies of the media and vehicle control are within the range of historical controls (Tables 3-6).

x. Data is presented as the number of TFT resistant mutant colonies/10^6 survivors and mean mutant frequency/10^6 survivors ± standard deviation (SD). For analysis, the mean mutant frequency/10^6 survivors of each test group, is compared to the mean mutant frequency/10^6 survivors of the vehicle control group. The individual plate count data were expressed as number of mutant colonies/10^6 survivors for each concentration (μg/ml) of test article (Appendix B).

E. Archives: All raw data generated at IITRI and a copy of the final report will be retained 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 consulted concerning 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. Cytotoxicity Assay (Initial Mutagenicity Assay): CuATSM/H_2ATSM was soluble in aqueous medium and was tested at up to 100 µg/ml in both the absence and presence of metabolic activation with a 4-hour exposure. Dose levels of 25 µg/ml and greater were excessively cytotoxic. There were no mutant colonies formed in any of the trifluorothymidine (TFT) culture plates, probably due to cytotoxicity (data not shown). However, the viable colony count plates (non-selective media, no TFT) did have colonies at dose levels of 25 µg/ml or less (dose levels of 25 µg/ml and greater were excessively toxic; i.e., the relative total growth (RTG) was less than 10%). From these plates a relative total growth was established for each dose level (see Table 1) and a dose range for an ensuing repeat initial assay was selected. In the repeat initial mutagenicity assay, in both the presence and absence of metabolic activation, a top dose level of 25 µg/ml was used to confirm the excessive toxicity at this dose level. The remaining dose levels in the repeat assay were 3.0, 5.0, 7.0, 10.0, 12.0, 15.0, 18.0, and 20.0 µg/ml. In the repeat initial mutagenicity assay in the non-activated test system dose levels of 15.0 µg/ml and greater demonstrated excessive cytotoxicity (the 20 µg/ml dose level was unanalyzable). Excessive cytotoxicity was indicated when the sample RTG was less than 10% relative to the vehicle control (cytotoxicity of 90%). The remaining dose levels ranged from 33% to 85% cytotoxicity relative to the vehicle control. In the S9-activated test system the 20.0 and 25.0 µg/ml dose levels demonstrated excessive cytotoxicity (the 25.0 µg/ml was unanalyzable). The remaining dose levels ranged from non-toxic to 71% cytotoxicity relative to the vehicle control.

In the initial range-finding assay, in both the absence and presence of metabolic activation the mutation frequency was not elevated by a factor of two or more times the vehicle mutation frequency in any of the dose levels tested.

In the repeat range-finding assay, in both the absence and presence of metabolic activation the mutation frequency was not elevated by a factor of two or more times the vehicle mutation frequency in any of the doses tested. The RTGs and the mutation frequencies of the remaining dose levels are listed in Tables 3 and 4.

Benzo[a]pyrene and methyl methanesulfonate were used as positive controls with and without S9, respectively. Both positive controls exhibited greater than a two-fold increase in $TK^{-/-}$ resistant colonies as compared to DMSO vehicle control articles.

B. Mutagenicity Assay (Definitive Study): In the definitive study CuATSM/H_2ATSM was tested at 0.50, 1.00, 3.00, 5.00, 10.00, 12.00 and 15.00 μg/ml in the absence of metabolic activation with a 24 hour exposure; and at 10.00, 12.00, 15.00, 18.00 and 20.00 μg/ml in the presence of S9-metabolic activation with a 4 hour exposure.

In the absence of metabolic activation, dose levels of 10.00 μg/ml or greater were not cloned due to excessive cytotoxicity. Cytotoxicity was observed during Day 1 and Day 2 cell counts (prior to cloning) as a decrease of 80% or greater in the relative suspension growth (RSG < 20%) relative to the vehicle control. The RTG in the absence of metabolic activation for the remaining dose levels (which were cloned) ranged from 50% to 12% of the vehicle control. In the presence of metabolic activation, all tested dose levels were analyzable and the cytotoxicity ranged from non-toxic to 72% relative to the vehicle control.

In the definitive mutagenicity assay, in both the absence and presence of metabolic activation, the mutation frequency was not elevated by a factor of at least two or more times the vehicle mutation frequency in any of the doses tested. The RTGs and the mutation frequencies for the definitive mutagenicity assay are listed in Table 5 and Table 6.

Benzo[a]pyrene and methyl methanesulfonate were used as positive controls with or without S9, respectively. Both positive control articles exhibited more than two-fold increases in L5178Y $TK^{-/-}$ or TFT resistant colonies as compared to the vehicle control article. In both assays, the positive and vehicle control articles exhibited positive and negative responses respectively, indicating the assay was sensitive and responsive to mutagens.

IV. DISCUSSION AND CONCLUSIONS

The solubility of CuATSM/H_2ATSM in dimethylsulfoxide (DMSO) permitted the conduct of *in vitro* studies at drug concentrations up to 100 μg/ml. In the initial mutagenicity assay, which also served as a cytotoxicity range-finding study, cytotoxicity was assessed by comparing the relative total growth (RTG) of the treated cultures to the vehicle control article cultures. CuATSM/H_2ATSM was initially tested up to 100 μg/ml in both the presence and absence of metabolic activation with a 4-hour exposure. There were no mutant colonies formed in any of the trifluorothymidine (TFT) culture plates, probably due to cytotoxicity (data not shown). However, the viable colony count plates (non-selective media, contained

no TFT) did have colonies at dose levels of 25 μg/ml or less (dose levels of 25 μg/ml and greater were excessively toxic; i.e., the relative total growth (RTG) was less than 10%). From these plates a relative total growth was established for each dose level and a dose range for an ensuing repeat initial assay was selected. In the repeat initial mutagenicity assay, in both the presence and absence of metabolic activation, a top dose level of 25 μg/ml was included to confirm the excessive toxicity previously observed at this dose level. The remaining dose levels in the repeat assay were 3.0, 5.0, 7.0, 10.0, 12.0, 15.0, 18.0, and 20.0 μg/ml (the 3.0 μg/ml dose level was not needed in the +S9 portion of the assay and therefore was not analyzed). In the repeat initial mutagenicity assay in the non-activated test system dose levels of 15.0 μg/ml and greater demonstrated excessive cytotoxicity (the 20 μg/ml dose level was unanalyzable). Excessive cytotoxicity was indicated when the sample RTG was less than 10% relative to the vehicle control (cytotoxicity of 90%). The remaining dose levels ranged from 33% to 85% cytotoxicity relative to the vehicle control. In the S9-activated test system the 20.0 and 25.0 μg/ml dose levels demonstrated excessive cytotoxicity (the 25.0 μg/ml was unanalyzable). The remaining dose levels ranged from non-toxic to 71% cytotoxicity relative to the vehicle control.

In the initial range-finding assay, in both the absence and presence of metabolic activation the mutation frequency was not elevated by a factor of two or more times the vehicle mutation frequency in any of the dose levels tested.

In the definitive mutagenicity assay, in the absence of metabolic activation with a 24-hour exposure, dose levels of 0.50, 1.00, 3.00, 5.00, 10.00, 12.00 and 15.00 μg/ml were tested. Dose levels of 10.00 μg/ml or greater were not cloned due to excessive cytotoxicity. Cytotoxicity was observed during Day 1 and Day 2 cell counts (prior to cloning) as a decrease of 80% or greater in the relative suspension growth (RSG < 20%) relative to the vehicle control. The RTG in the absence of metabolic activation for the remaining dose levels (which were cloned) ranged from 50% to 12% of the vehicle control. In the presence of metabolic activation with a 4-hour exposure dose levels of 10.00, 12.00, 15.00, 18.00 and 20.00 μg/ml were tested. All tested dose levels were analyzable and the cytotoxicity ranged from non-toxic to 72% relative to the vehicle control.

In the definitive mutagenicity assay, in both the absence and presence of metabolic activation, none of the tested dose levels resulted in an increase in mutant frequency that was at least a

2-fold increase relative to the vehicle control values. Therefore all tested dose levels of CuATSM/H_2ATSM were negative for mutagenicity.

CuATSM/H_2ATSM was concluded to be non-mutagenic in the mouse lymphoma assay.

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VI. QUALITY ASSURANCE STATEMENT

Study Title: Evaluation of the Potential Mutagenic Activity of CuATSM/H_2ATSM (NSC-729307) in the Mouse Lymphoma Assay

Project number: 2073-002-001-001

Study Director: Patrick T. Curry, Ph.D.

This Study has been inspected and the report has been audited by the IITRI Quality Assurance Unit in compliance with the U.S. Food and Drug Administration (FDA) Good Laboratory Practice Regulations as set forth in the *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 the inspection findings were reported:

	Findings Reported to:	
Inspection Dates	Study Director	Management
May 24, 2004	May 24, 2004	May 27, 2004
June 15-16, 2004	June 16, 2004	June 28, 2004
June 16, 2004	June 16, 2004	June 24, 2004
June 16, 2004	June 16, 2004	June 24, 2004
October 5-8, 2004	October 8, 2004	October 19, 2004
October 19, 2004	October 20, 2004	October 22, 2004
January 14, 2005	January 18, 2005	January 18, 2005

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

1-18-05
Date

VII. TABLES

EVALUATION OF THE POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM (NSC-D729307) IN THE MOUSE LYMPHOMA ASSAY

Table 1

Summary of Cytotoxicity Assay (Initial Mutagenicity Assay) - without S9
(4 hour exposure)

Test Article (Concentration)	%Relative Cloning Efficiency (RCE) (Mean ± SD)	%Relative Total Growth (RTG) (Mean ± SD)
Media Control	100 ± 14.75	99 ± 14.60
Vehicle Control (0.0%)	99 ± 6.07	109 ± 7.29
Methyl Methanesulfonate (26.8 µg/ml)	30 ± 0.87	14 ± 0.42
CuATSM/H_2ATSM (0.25 µg/ml)	90 ± 6.51	74 ± 32.28
CuATSM/H_2ATSM (0.50 µg/ml)	89 ± 32.54	82 ± 15.25
CuATSM/H_2ATSM (1.00 µg/ml)	101 ± 18.65	102 ± 43.10
CuATSM/H_2ATSM (3.00 µg/ml)	88 ± 2.60	101 ± 8.60
CuATSM/H_2ATSM (5.00 µg/ml)	60 ± 11.71	45 ± 13.80
CuATSM/H_2ATSM (10.00 µg/ml)	79 ± 9.11	49 ± 25.45
CuATSM/H_2ATSM (25.00 µg/ml)	21 ± 2.68	1 ± 0.13
CuATSM/H_2ATSM (30.00 µg/ml)	--[a] --	-- --
CuATSM/H_2ATSM (40.00 µg/ml)	--[a] --	-- --
CuATSM/H_2ATSM (100.00 µg/ml)	--[a] --	-- --

[a] -- = Not analyzable due to excessive toxicity

EVALUATION OF THE POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM (NSC-D729307) IN THE MOUSE LYMPHOMA ASSAY

Table 2

Summary of Cytotoxicity Assay (Initial Mutagenicity Assay) - with S9
(4 hour exposure)

Test Article (Concentration)	%Relative Cloning Efficiency (RCE) (Mean ± SD)	%Relative Total Growth (RTG) (Mean ± SD)
Media Control	100 ± 7.50	100 ± 6.08
Vehicle Control (0.0%)	119 ± 3.00	119 ± 13.13
Methyl Methanesulfonate (26.8 µg/ml)	53 ± 2.50	24 ± 3.36
CuATSM/H_2ATSM (0.25 µg/ml)	118 ± 1.50	113 ± 8.94
CuATSM/H_2ATSM (0.50 µg/ml)	111 ± 4.50	111 ± 14.75
CuATSM/H_2ATSM (1.00 µg/ml)	99 ± 5.50	98 ± 1.96
CuATSM/H_2ATSM (3.00 µg/ml)	95 ± 11.99	71 ± 27.76
CuATSM/H_2ATSM (5.00 µg/ml)	92 ± 21.49	68 ± 28.09
CuATSM/H_2ATSM (10.00 µg/ml)	110 ± 6.00	73 ± 3.21
CuATSM/H_2ATSM (25.00 µg/ml)	22 ± 2.50	3 ± 1.75
CuATSM/H_2ATSM (30.00 µg/ml)	--[a]	-- --
CuATSM/H_2ATSM (40.00 µg/ml)	--[a]	-- --
CuATSM/H_2ATSM (100.00 µg/ml)	--[a]	-- --

[a] -- = Not analyzable due to excessive toxicity

EVALUATION OF THE POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM (NSC-D729307) IN THE MOUSE LYMPHOMA ASSAY

Table 3

Summary of Repeat Cytotoxicity Assay (Repeat Initial Mutagenicity Assay) - without S9
(4 hour exposure)

Test Article (Concentration)	%Relative Cloning Efficiency (RCE) (Mean ± SD)	%Relative Total Growth (RTG) (Mean ± SD)	Mutation Frequency (TFT Mutant Colonies / 10^6 Survivors) (Unsized Colonies) (Mean ± SD)
Media Control	100 ± 20.20	101 ± 32.97	80 ± 13.87
Vehicle Control (1.0%)	100 ± 4.14	98 ± 8.30	92 ± 12.73
Methyl Methanesulfonate (26.8 µg/ml)	87 ± 23.11	75 ± 8.64	286 ± 39.65
CuATSM/H_2ATSM (3.00 µg/ml)	103 ± 5.52	67 ± 1.13	91 ± 10.26
CuATSM/H_2ATSM (5.00 µg/ml)	105 ± 13.45	55 ± 15.59	83 ± 6.77
CuATSM/H_2ATSM (7.00 µg/ml)	95 ± 3.10	68 ± 13.28	94 ± 11.75
CuATSM/H_2ATSM (10.00 µg/ml)	53 ± 3.79	28 ± 2.94	135 ± 5.91
CuATSM/H_2ATSM (12.00 µg/ml)	55 ± 3.45	15 ± 1.22	138 ± 1.13
CuATSM/H_2ATSM (15.00 µg/ml)	47 ± 1.38	7 ± 0.53	131 ± 3.47
CuATSM/H_2ATSM (18.00 µg/ml)	30 ± 3.10	3 ± 0.16	158 ± 18.96
CuATSM/H_2ATSM (20.00 µg/ml)	--[a] --	-- --	-- --
CuATSM/H_2ATSM (25.00 µg/ml)	--[a] --	-- --	-- --

[a] -- = Not analyzable due to excessive toxicity

EVALUATION OF THE POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM (NSC-D729307) IN THE MOUSE LYMPHOMA ASSAY

Table 4

Summary of Repeat Cytotoxicity Assay (Repeat Initial Mutagenicity Assay) - with S9
(4 hour exposure)

Test Article (Concentration)	%Relative Cloning Efficiency (RCE) (Mean ± SD)	%Relative Total Growth (RTG) (Mean ± SD)	Mutation Frequency (TFT Mutant Colonies / 10^6 Survivors) (Unsized Colonies) (Mean ± SD)
Media Control	100 ± 1.47	100 ± 2.77	77 ± 10.71
Vehicle Control (1.0%)	100 ± 3.92	100 ± 2.51	87 ± 2.62
Benzo[a]Pyrene (2.0 µg/ml)	38 ± 3.62	25 ± 2.82	530 ± 31.15
CuATSM/H_2ATSM (5.00 µg/ml)	87 ± 1.81	102 ± 6.49	84 ± 6.6
CuATSM/H_2ATSM (7.00 µg/ml)	82 ± 1.21	100 ± 23.08	93 ± 7.48
CuATSM/H_2ATSM (10.00 µg/ml)	71 ± 1.51	93 ± 12.52	89 ± 12.54
CuATSM/H_2ATSM (12.00 µg/ml)	83 ± 11.16	75 ± 10.04	87 ± 13.86
CuATSM/H_2ATSM (15.00 µg/ml)	78 ± 0.60	51 ± 15.98	95 ± 6.19
CuATSM/H_2ATSM (18.00 µg/ml)	65 ± 3.02	29 ± 9.23	96 ± 15.88
CuATSM/H_2ATSM (20.00 µg/ml)	46 ± 7.54	8 ± 2.03	142 ± 14.04
CuATSM/H_2ATSM (25.00 µg/ml)	--[a] --	-- --	-- --

[a] -- = Not analyzable due to excessive toxicity

EVALUATION OF THE POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM (NSC-D729307) IN THE MOUSE LYMPHOMA ASSAY

Table 5

Summary of Mutagenicity Assay (Confirmatory Mutagenicity Assay) - without S9
(24 hour exposure)

Test Article (Concentration)	%Relative Cloning Efficiency (RCE) (Mean ± SD)	%Relative Total Growth (RTG) (Mean ± SD)	Mutation Frequency (TFT Mutant Colonies / 10^6 Survivors) (Unsized Colonies) (Mean ± SD)
Media Control	100 ± 2.54	101 ± 20.94	78 ± 7.06
Vehicle Control (1.0%)	100 ± 2.46	77 ± 9.18	71 ± 25.42
Methyl Methanesulfonate (26.8 µg/ml)	47 ± 5.91	22 ± 4.08	480 ± 39.71
CuATSM/H_2ATSM (0.50 µg/ml)	63 ± 6.90	50 ± 2.61	77 ± 38.28
CuATSM/H_2ATSM (1.00 µg/ml)	71 ± 5.42	59 ± 7.32	70 ± 10.00
CuATSM/H_2ATSM (3.00 µg/ml)	44 ± 2.96	17 ± 2.92	80 ± 27.86
CuATSM/H_2ATSM (5.00 µg/ml)	37 ± 4.93	12 ± 2.78	100 ± 23.98
CuATSM/H_2ATSM (10.00 µg/ml)	--[a] --	-- --	-- --
CuATSM/H_2ATSM (12.00 µg/ml)	--[a] --	-- --	-- --
CuATSM/H_2ATSM (15.00 µg/ml)	--[a] --	-- --	-- --

[a] -- = Not analyzable due to excessive toxicity

EVALUATION OF THE POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM (NSC-D729307) IN THE MOUSE LYMPHOMA ASSAY

Table 6

Summary of Mutagenicity Assay (Confirmatory Mutagenicity Assay) - with S9
(4 hour exposure)

Test Article (Concentration)	%Relative Cloning Efficiency (RCE) (Mean ± SD)	%Relative Total Growth (RTG) (Mean ± SD)	Mutation Frequency (TFT Mutant Colonies / 10^6 Survivors) (Unsized Colonies) (Mean ± SD)
Media Control	100 ± 6.69	100 ± 4.61	90 ± 14.52
Vehicle Control (1.0%)	100 ± 4.70	100 ± 13.19	91 ± 6.44
Benzo[a]Pyrene (2.0 µg/ml)	46 ± 17.36	20 ± 8.16	799 ± 6.77
CuATSM/H_2ATSM (10.00 µg/ml)	78 ± 19.53	80 ± 5.11	104 ± 24.36
CuATSM/H_2ATSM (12.00 µg/ml)	57 ± 6.87	57 ± 11.31	130 ± 11.99
CuATSM/H_2ATSM (15.00 µg/ml)	107 ± 15.55	93 ± 14.40	84 ± 6.82
CuATSM/H_2ATSM (18.00 µg/ml)	79 ± 24.60	43 ± 18.74	121 ± 2.65
CuATSM/H_2ATSM (20.00 µg/ml)	60 ± 10.13	28 ± 7.17	145 ± 31.53

VIII. APPENDICES

Appendix A: Protocol and Protocol Amendment

EVALUATION OF THE POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM (NSC-D729307) IN THE MOUSE LYMPHOMA ASSAY

SPONSOR:

Toxicology and 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 R. Glaze, Ph.D.

CONTRACT NUMBER: N 01-CM-42202

CONTRACTOR:

IIT Research Institute (IITRI)
Life Sciences Group
10 West 35th Street
Chicago, IL 60616

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

STUDY DIRECTOR: Patrick Curry, Ph.D.

PROPOSED SCHEDULE:

Start: June 14, 2004

Draft Report: August 27, 2004

I. OBJECTIVE

The objective of this study is to determine the potential mutagenic activity of copper-diacetyl-bis(N^4-methylthiosemicarbazone)/diacetyl-bis-(N^4-methyl thiosemicarbazone (CuATSM/H_2ATSM; NSC-D729307) using the mouse lymphoma assay with and without metabolic activation.

II. MATERIALS AND METHODS:

A. Test and Control Articles:

1. Name of Test Article.

Copper-diacetyl-bis (N^4-methylthiosemicarbazone)/diacetyl-bis-(N^4-methylthiosemicarbazone (CuATSM/H_2ATSM; NSC-D729307)

2. Name of Control Articles:

Control Article: Dimethylsulfoxide (DMSO)

Positive Controls:

(-S9): Methyl methanesulfonate (MMS)
(+S9): Benzo(α)pyrene (BaP)

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

a. Test Article

The test article for this study is CuATSM/H_2ATSM; NSC-D729307, an imaging agent. The test article will be dissolved in DMSO to formulate the dosing solutions. The dosing solutions will be stored at room temperature and used as quickly as practical after formulation. Compound identity, strength, quality, stability, and purity as well as documentation of methods of synthesis, fabrication or derivation are the responsibility of NCI. No archival sample of the test article will be maintained by IITRI. The Sponsor is responsible for maintenance of an archival sample of the test article. The unused test article will be returned to the Sponsor following completion of the study.

b. Control Articles

The positive controls will be benzo (α) pyrene (BaP) for the S9-activated system and methyl methanesulfonate (MMS) for the non-activated system. Both positive controls will be dissolved in dimethylsulfoxide (DMSO) and stored frozen until use; at which time they will be thawed and diluted to dosing stock solutions. The final concentration of BaP will be

approximately 2.0 µg/ml, and the final concentration of MMS will be approximately 26 µg/ml. The exposure period for each of the positive controls will be 4 hours. The positive control is not expected to contain any contaminants and is assumed to be stable under the conditions of administration. Concentration verification of the positive control substances will not be conducted. The positive controls will be characterized by product labels and by performance in the assay.

The vehicle, DMSO, will be administered by addition to the culture medium and will not exceed 1% of the final volume of the treatment medium. Vehicle cultures will be dosed concurrently and handled identically to test article-treated cultures.

4. Stability and Storage:

a. Test Article:

The test article will be stored frozen, protected from light.

B. Test System:

Cell Strain, Supplier and Test System Justification:

The mutagenicity of the test article, CuATSM/H_2ATSM, will be determined in mouse lymphoma L5178Y $TK^{+/-}$ cells (American Type Culture Collection, ATCC, Manassas, VA). The L5178Y $TK^{+/-}$ cell line when cultured *in vitro* with mutagenic/carcinogenic agents is mutated to the $TK^{-/-}$ genotype. The mutant cells are resistant to the cytotoxic effects of pyrimidine analogue trifluorothymidine (TFT), whereas the wild type cells are sensitive. Thus, $TK^{-/-}$ homozygous cells proliferate and grow into round colonies when cloned in soft agar medium containing TFT. The mouse lymphoma assay is a mammalian cell gene mutation assay and is used to detect forward gene mutations induced by chemical agents at the heterozygous thymidine kinase locus ($TK^{+/-}$) (Clive *et al.*, 1983, 1995; Turner *et al.*, 1984; and Moore *et al.*, 2000).

C. Experimental Design

The test article will be administered to the L5178Y $TK^{+/-}$ cell cultures by addition of dosing solutions to the culture medium. The test article will be dissolved in the vehicle (DMSO) such that the dosing solution concentrations are 100-fold in excess of the final target concentrations. A 100 µl aliquot of the respective dosing solution will then be added to approximately 9.9 ml of treatment medium to achieve the final target concentrations. Exposure of the cells to the test article will be for 4 hours in the –S9 and the +S9 test systems. An additional exposure of 24 hours will also be employed in the –S9 test system.

The cytotoxicity of the test article will be assayed with and without metabolic activation (S9). The test article will be tested up to 100 µg/ml. This upper dose limit is based on solubility of the test article/DMSO formulation in an aqueous milieu, and will include testing of at least one precipitating concentration. The cytotoxicity of up to 10 doses of the test article will be determined. If the test article is cytotoxic, then the dose range will be selected in such a way that the dose range will exhibit minimal cytotoxicity to approximately 90% cytotoxicity. The cytotoxicity assay will include appropriate controls (positive and vehicle), and will be conducted with replicate cultures for each test condition. If there are at least 4 dose groups with relative suspension growth (RSG) values that fall between minimal cytotoxicity and approximately 90% cytotoxicity then the cultures will be cloned for cloning efficiency and trifluorothymidine (TFT) mutant selection. Therefore the cytotoxicity assay may become the initial mutagenicity assay if the toxicity profile of several doses falls within the optimal toxicity range. If there is no apparent cytotoxicity then the dose range will include at least one precipitating concentration.

1. Mouse Lymphoma Assay Procedures:

a. Media and Reagent Preparation

All media and reagents will be prepared using purified distilled ASTM type 1 water. Glassware will be sterilized by autoclaving; media and reagent solutions will be sterilized by passing through 0.2 µm filters. The suppliers of all reagents and media will be recorded in the raw data.

The L5178Y cells ($TK^{+/-}$, Clone 3.7.2C) will be maintained in log phase growth by serial subculturing in a shaker incubator at 37°C. The cells will be cultured in RPMI-1640 media supplemented with 25 mM HEPES and 2 mM L-glutamine, 8.2% Horse Serum, 8.1mg/ml Pluronic F-68, 17.9 units/ml Penicillin and 17.9 µg/ml Streptomycin, and 0.20 mg/ml Sodium Pyruvate (this formulation is referred to as complete medium). During treatment with test article, the horse serum (heat inactivated) concentration will be reduced to approximately 4%.

The cloning media consists of 2.36 g/l agar in complete RPMI-1640 (complete medium with 10% heat inactivated horse serum). The molten agar is added to the complete medium and dispensed into 125 ml and 250 ml culture flasks and stored at 37°C until use.

For metabolic activation of promutagens, Aroclor-1254 induced rat liver post-mitochondrial S9 fraction (Molecular Toxicology Inc., Boone, NC) will be used. The S9 mixture consists of 25% S9 (5% final concentration), 5.0 mmol/l glucose-6-phosphate monosodium, 0.8 mmol/l NADP and RPMI-1640 media. The S9 mix will be filter sterilized by passage through a 0.45 µm filter and kept in an ice bath until used.

b. Assay Procedure

Treatment: Test article concentrations for the cytotoxicity assessment are listed in Table 1. (Based on the actual solubility of the test article the test concentrations may vary from those listed.) In the cytotoxicity experiment, up to 10 doses of the test article up to 100 µg/ml will be evaluated for cytotoxicity with and without S9. If the relative suspension growth (RSG) is above 10% with these doses, the cultures will be cloned for cloning efficiency and trifluorothymidine (TFT) mutant selection and the cytotoxicity will become the initial mutagenicity assay. For the confirmatory mutagenicity assay 5-8 doses of test article will be tested (Table 2 - Based on the actual cytotoxicity of the test article in the initial trial the test concentrations may vary from those listed.).

Table 1. Treatment of Samples – Cytotoxicity Assay (Initial Mutagenicity Assay)

Sample Groups	Percent S9
Control Article – DMSO	0 or 5
Positive Control – BaP (with S9)	1
Positive Control - MMS (without S9)	0
Test Article Concentration 100 µg/ml	5 or 0
Test Article Concentration 40 µg/ml	5 or 0
Test Article Concentration 30 µg/ml	5 or 0
Test Article Concentration 25 µg/ml	5 or 0
Test Article Concentration 10 µg/ml	5 or 0
Test Article Concentration 5 µg/ml	5 or 0
Test Article Concentration 3 µg/ml	5 or 0
Test Article Concentration 1 µg/ml	5 or 0
Test Article Concentration 0.5 µg/ml	5 or 0
Test Article Concentration 0.25 µg/ml	5 or 0

Each culture tube will be labeled with the project number, date, test article identification, metabolic activation system, and test article concentration (vehicle, or positive control). The culture tubes will contain 7.0 ml of cell suspension containing 8.6 x 10^5 cells/ml suspended in a 1:1:2 mixture of complete medium, RPMI-1640 (RPMI), and complete media collected from the earlier cultured cell suspension (conditioned media). To these cells, 2 ml of either S9 mix (4 hour exposure only) or RPMI will be added. Then 1.0 ml RPMI, test article, positive, or vehicle control will be added (changing the final cell concentration to 6.0x10^5 cells/ml). Following addition of the test article, tubes will be gassed with 5% CO_2 in air and incubated for 4 hours at 37°C in a roller drum set at 40-45 orbits/minute. (In the confirmatory

mutagenicity assay, the non-activated test system will employ a 24 hour exposure time for the cells and the density of the cell suspension in the culture tubes will be 3 X 10^5 cells/ml.) At the end of the incubation period, the cells will be pelleted and washed with RPMI and finally re-suspended in 10 ml of complete medium. Cells will be counted with hemacytometer or a Coulter counter and the final cell concentration will be adjusted to 2.0 x10^5 cells/ml in 20 ml of complete media. The tubes will again be gassed and transferred to a roller drum for 2 days. The two days of post-treatment culture are known as the expression period.

Table 2. Treatment of Samples – Mutagenicity Assay (Confirmatory Mutagenicity)

Sample Groups	Percent S9
Control Article – DMSO	0 or 5
Positive Control – BaP (with S9)	1
Positive Control – MMS (without S9)	0
Test Article Concentration 30 mg/ml	5 or 0
Test Article Concentration 20 mg/ml	5 or 0
Test Article Concentration 10 mg/ml	5 or 0
Test Article Concentration 5 mg/ml	5 or 0
Test Article Concentration 3 mg/ml	5 or 0
Test Article Concentration 1 mg/ml	5 or 0
Test Article Concentration 0.5 mg/ml	5 or 0
Test Article Concentration 0.25 mg/ml	5 or 0

Expression Period: At approximately 24 hours after treatment initiation (or 48 hours for the 24 hour –S9 exposure), the cultures will be counted and diluted to a concentration of 2.0x10^5 cells/ml with fresh complete medium and returned to the roller drum. This process will be repeated approximately 24 hours later; however, the cells will be re-suspended at a concentration of approximately 3.0 x 10^5 cells/ml.

Cloning: In this study, cloning will be done approximately 48 hours after treatment initiation for the 4 hour exposure cultures (+/-S9) and approximately 72 hours after treatment initiation for the 24 hour continuous exposure cultures (-S9). On this day, cells will be counted and approximately 3 x 10^6 cells will be suspended in 10 ml of media and incubated in a roller drum for no less than 30 min. The cells are then centrifuged and all but approximately 1-2 ml of the supernatant is discarded. Each culture will be suspended in 100 ml of cloning media (complete medium containing 10% heat inactivated horse serum with

0.24% soft agar). A portion of this suspension will be diluted to 600 cells/100 ml of cloning media for viable count (VC). Then, the first suspension is supplemented with 1 µg/ml TFT, mixed in the shaker incubator, and dispensed into 3 (20 x 100 mm) petri dishes, or plates, at approximately 1 x 10^6 cells per plate. The VC suspension will also be agitated in the shaker incubator, and dispensed into 3 plates, at a concentration of approximately 200 cells/plate. The dishes will be allowed to gel for 10-15 minutes in a refrigerator (2-8°C) and then they will be transferred to an incubator at 37°C for 10-14 days for mutant colony formation. The mutant colonies in each plate will be counted with an automatic colony counter. Both large and small colonies will be counted in each plate and the data expressed as the sum of both types of colonies. The data will be calculated using the following equations:

Relative Suspension Growth (RSG) Day 1:

$$\frac{\text{Cell concentration of individual culture}}{\text{Average concentration of vehicle culture}} \times 100$$

Relative Suspension Growth (RSG) Day 2:

$$\frac{\text{Cell concentration of individual culture Day 2)}}{\text{Average concentration of vehicle culture Day 2}} \times 100$$

Cumulative RSG:

$$\frac{\text{Day 1 RSG X Day 2 RSG}}{100}$$

Cloning Efficiency (CE):

$$\frac{\text{Average no. of colonies in viable count (VC) plates}}{\text{Total no. of cells seeded per VC plate}} \times 100$$

Relative Cloning Efficiency (RCE):

$$\frac{\text{Average no. of colonies in test VC plates}}{\text{Average no. of colonies in vehicle control VC plates}} \times 100$$

Relative Total Growth (RTG):

$$\frac{\text{Day 2 cumulative RSG X RCE}}{100}$$

Mutation Frequency (MF):

$$\frac{\text{Average no. of colonies per TFT plate}}{\text{Cloning efficiency (CE)}} \times 100$$

2. Results and Statistical Analysis:

a. Criteria for a Valid Assay

The assay will be considered valid only if: (1) the positive controls exhibit a mutant frequency approximately twice that of the vehicle control with an RTG of 10% or greater, (2) the cloning efficiency of the vehicle cultures is 60% or greater, and (3) the mutant frequencies of the media and vehicle controls are within the range of historical controls.

b. Results

TFT resistant mutation frequency, in the presence and absence of metabolic activation, is expressed as mutant colonies per 10^6 surviving cells. The mean, along with its associated standard deviation will be calculated for each concentration. The colonies per plate and the mutant frequencies will be reported for the vehicle, the positive controls and each dose level.

c. Evaluation of Mutagenicity

A response induced by the test article will be considered positive (mutagenic) under the following conditions: (1) mutant frequency increases in a concentration-related manner, and/or (2) the mutant frequency is approximately twice that of vehicle with relative total growth (RTG) not less than 10%. In the absence of concentration-related increase, the response will be considered positive under the following condition: At least one concentration of the test article induced approximately twice the mutant frequency of vehicle control with RTG above 10%.

III. QUALITY ASSURANCE:

A. Type of Study and Regulatory Compliance

This is a non-clinical 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 for the changes will be documented, signed and dated by the Study Director, Principal Investigator, 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

All data documenting experimental details and study procedures and observations will be recorded and maintained as raw data. The raw data and the report will be audited by the IIITRI 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. The Sponsor is responsible for all costs associated with the storage of these materials beyond one year from the issuance of the final report and for any costs associated with the shipment of these materials to the Sponsor or to any other facility designated by the Sponsor. 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 the request of the Sponsor. 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 48 working days after the completion of the in-life phase of the study. 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 be limited to: (a) a cover page which will include title, contract number, author, 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; a statement of GLP compliance; and (g) a statement of where the raw data records, reports and samples are stored.

REFERENCES:

a. Clive, D.C. *et al.,* 1983. Specific gene mutations in L5178Y cells in culture. A report of the U.S. Environmental Protection Agency Gene-Tox Program. Mutation research 115:225-251.

b. Clive, D.C. *et al.,* 1995. Consensus agreement regarding protocol issues discussed during the mouse lymphoma workshop: Portland, Oregon, May 7, 1994. Environmental and Molecular Mutagenesis 25:165-168.

c. FDA Redbook 2000, Toxicological principles for the safety of food ingredients: IV.C.1.c. Mouse lymphoma thymidine kinase gene mutation assay, October, 2001.

d. Moore, M.M., *et al.,* 2000. Mouse lymphoma thymidine kinase locus gene mutation assay. International workshop on genotoxicity procedures workgroup report. Environmental and Molecular Mutagenesis 35:185-190.

e. Turner, N.T., *et al.,* 1984. Procedures for the L5178Y/TK+/- → TK -/- mouse lymphoma cell mutagenicity assay. Kilby, B.J., Legator, M., Nichols, W. and Ramel, C. (Eds), Handbook of Mutagenicity Test Procedures, pp239-267, Elsevier Science Publishers, New York, USA.

Protocol Approvals:

Study Director: Patricia T. Cheng 5/25/04 (Date)

Principal Investigator: [signature] 5/25/04 (Date)

Project Officer: [signature] 6/7/04 (Date)

IIT RESEARCH INSTITUTE
PROTOCOL AMENDMENT #1

Title: Evaluation of the Potential Mutagenic Activity of CuATSM/H_2ATSM (NSC-D729307) in the Mouse Lymphoma Assay

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

The following indicates changes to the protocol:

Effective Date: June 10, 2004

Section: Header Throughout the Protocol

Amendment: Amend the IITRI Project No. to read: 2073-002-001-001

Reason for Amendment: To correct an inadvertent typographical error throughout the protocol.

Section II. A. 1: Name of Test Article

Amendment: Amend the test article name to include the bolded parenthesis as follows:

Copper-diacetyl-bis (N^4-methylthiosemicarbazone)/diacetyl-bis-(N^4-methylthiosemicarbazone) (CuATSM/H_2ATSM;NSC-D729307)

Reason for Amendment: To include the parenthesis as part of the test article identification.

Section II. C. 1.b: Assay Procedure

Amendment: Amend the volumes and cell density in the second and fourth sentences after Table 1 in this section as follows:

Second Sentence:

The culture tubes will contain 7.9 ml of cell suspension containing 7.6 x 10^5 cells/ml suspended in a 1:1:2 mixture of complete medium, RPMI-1640 (RPMI), and complete media collected from the earlier cultured cell suspension (conditioned media).

Fourth Sentence:

Then 0.1 ml RPMI, test article, positive, or vehicle control will be added (changing the final cell concentration to $6.0x10^5$ cells/ml).

Reason for Amendment: To adjust the volumes/cell densities to values used with an organic vehicle.

Section II. C. 1.b: Table 2

Amendment: Amend the concentration units from mg/ml to µg/ml.

Reason for Amendment: To correct an inadvertent typographical error in the Table.

Amendment Approvals:

Study Director: Patrick T. Cronin 6/29/04 (Date)

Principal Investigator: [signature] 6/29/04 (Date)

Project Officer: [signature] 8/14/04 (Date)

Appendix B: Individual Assay Data

EVALUATION OF THE POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM (NSC-D729307) IN THE MOUSE LYMPHOMA ASSAY

Appendix B Table B-1

Cytotoxicity Assay (Initial Mutagenicity Assay) - without S9

Colony Counts per Plate and Cloning Efficiency

Test Article (Concentration)	Sample ID	Cells Seeded	Viable Count Plates A	B	C	Mean	±	SE	Cloning Efficiency (CE)[a]
Media Control	1	200	170	185	184	180	±	5	90
	2	200	146	145	147	146	±	1	73
Vehicle Control (0.0%)	1	200	132	148	181	154	±	14	77
	2	200	179	134	192	168	±	17	84
Methyl Methanesulfonate (26.8 µg/ml)	1	200	44	48	52	48	±	2	24
	2	200	45	51	54	50	±	3	25
CuATSM/H_2ATSM (0.25 µg/ml)	1	200	158	145	162	155	±	5	77
	2	200	148	122	149	140	±	9	70
CuATSM/H_2ATSM (0.50 µg/ml)	1	200	107	115	103	108	±	3	54
	2	200	185	171	194	183	±	7	91
CuATSM/H_2ATSM (1.00 µg/ml)	1	200	141	147	141	143	±	2	71
	2	200	189	204	166	186	±	11	93
CuATSM/H_2ATSM (3.00 µg/ml)	1	200	160	138	126	141	±	10	70
	2	200	146	131	164	147	±	10	73
CuATSM/H_2ATSM (5.00 µg/ml)	1	200	95	88	71	85	±	7	42
	2	200	85	118	132	112	±	14	56
CuATSM/H_2ATSM (10.00 µg/ml)	1	200	152	132	135	140	±	6	70
	2	200	124	127	107	119	±	6	59
CuATSM/H_2ATSM (25.00 µg/ml)	1	188	32	42	32	35	±	3	19
	2	200	28	34	32	31	±	2	15
CuATSM/H_2ATSM (30.00 µg/ml)	1	200	--[b]	--	--	--		--	--
	2	200	--[b]	--	--	--		--	--
CuATSM/H_2ATSM (40.00 µg/ml)	1	200	--[b]	--	--	--		--	--
	2	200	--[b]	--	--	--		--	--
CuATSM/H_2ATSM (100.00 µg/ml)	1	200	--[b]	--	--	--		--	--
	2	200	--[b]	--	--	--		--	--

[a] Cloning Efficiency = (Mean number of colonies in viable count plates ÷ Total number of cells seeded) x 100
[b] -- = Not analyzable due to excessive cytotoxicity

EVALUATION OF THE POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM (NSC-D729307) IN THE MOUSE LYMPHOMA ASSAY

Appendix B Table B-2

Cytotoxicity Assay (Initial Mutagenicity Assay) - without S9

Relative Suspension Growth

Test Article (Concentration)	Sample ID	Day 1 Cell Concentration (cells/ml)	Day 1 %Relative Suspension Growth (RSG)[c]	Day 2 Cell Concentration (cells/ml)	Day 2 %Relative Suspension Growth (RSG)[c]	Cumulative %Relative Suspension Growth[d]
Media Control	1	$8.73x10^5$	90	$1.29x10^6$	110	99
	2	$1.07x10^6$	110	$1.05x10^6$	90	99
Vehicle Control (0.0%)	1	$1.08x10^6$	112	$1.16x10^6$	107	120
	2	$1.05x10^6$	108	$1.00x10^6$	93	100
Methyl Methanesulfonate (26.8 µg/ml)	1	$7.10x10^5$	73	$7.13x10^5$	66	48
	2	$6.49x10^5$	67	$7.63x10^5$	71	48
CuATSM/H_2ATSM (0.25 µg/ml)	1	$9.88x10^5$	102	$1.10x10^6$	102	104
	2	$8.54x10^5$	88	$7.69x10^5$	71	62
CuATSM/H_2ATSM (0.50 µg/ml)	1	$1.01x10^5$	105	$1.06x10^6$	98	103
	2	$7.56x10^5$	78	$1.11x10^6$	103	80
CuATSM/H_2ATSM (1.00 µg/ml)	1	$9.98x10^5$	103	$8.90x10^5$	82	84
	2	$1.06x10^6$	110	$1.15x10^6$	107	118
CuATSM/H_2ATSM (3.00 µg/ml)	1	$1.07x10^6$	110	$1.08x10^6$	100	110
	2	$1.02x10^6$	105	$1.22x10^6$	113	119
CuATSM/H_2ATSM (5.00 µg/ml)	1	$1.03x10^6$	107	$6.93x10^5$	64	68
	2	$9.85x10^5$	102	$8.42x10^5$	78	80
CuATSM/H_2ATSM (10.00 µg/ml)	1	$7.70x10^5$	79	$1.11x10^6$	102	81
	2	$5.94x10^5$	61	$8.25x10^5$	76	46
CuATSM/H_2ATSM (25.00 µg/ml)	1	$1.84x10^5$	19	$2.82x10^5$	26	5
	2	$2.16x10^5$	22	$3.33x10^5$	31	7
CuATSM/H_2ATSM (30.00 µg/ml)	1	$1.75x10^5$	18	--[b]	--	--
	2	$1.73x10^5$	18	--[b]	--	--
CuATSM/H_2ATSM (40.00 µg/ml)	1	$1.48x10^5$	15	--[b]	--	--
	2	$1.53x10^5$	16	--[b]	--	--
CuATSM/H_2ATSM (100.00 µg/ml)	1	$1.21x10^5$	12	--[b]	--	--
	2	$1.27x10^5$	13	--[b]	--	--

[b] -- = Not analyzable due to excessive cytotoxicity

[c] Methyl Methanesulfonate and test articles are relative to the vehicle control

[d] Cumulative %Relative Suspension Growth = [(Day 1 RSG) x (Day 2 RSG)] ÷ 100 (original un-rounded values were used for calculations)

EVALUATION OF THE POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM (NSC-D729307) IN THE MOUSE LYMPHOMA ASSAY

Appendix B Table B-3

Cytotoxicity Assay (Initial Mutagenicity Assay) - with S9

Colony Counts per Plate and Cloning Efficiency

Test Article (Concentration)	Sample ID	Cells Seeded	Viable Count Plates A	B	C	Mean	±	SE	Cloning Efficiency (CE)[a]
Media Control	1	200	136	126	139	134	±	4	67
	2	200	142	170	135	149	±	11	74
Vehicle Control (0.0%)	1	200	160	169	170	166	±	3	83
	2	200	178	168	169	172	±	3	86
Methyl Methanesulfonate (26.8 µg/ml)	1	200	75	79	77	77	±	1	38
	2	200	70	70	75	72	±	2	36
CuATSM/H_2ATSM (0.25 µg/ml)	1	200	164	168	164	165	±	1	82
	2	200	189	148	168	168	±	12	84
CuATSM/H_2ATSM (0.50 µg/ml)	1	200	156	152	150	153	±	2	76
	2	200	162	160	165	162	±	2	81
CuATSM/H_2ATSM (1.00 µg/ml)	1	200	150	129	126	135	±	8	67
	2	200	156	142	141	146	±	5	73
CuATSM/H_2ATSM (3.00 µg/ml)	1	200	155	139	147	147	±	5	73
	2	200	120	127	122	123	±	2	61
CuATSM/H_2ATSM (5.00 µg/ml)	1	200	105	127	91	108	±	10	54
	2	200	147	143	164	151	±	6	75
CuATSM/H_2ATSM (10.00 µg/ml)	1	200	163	167	156	162	±	3	81
	2	200	160	138	153	150	±	6	75
CuATSM/H_2ATSM (25.00 µg/ml)	1	200	29	32	25	29	±	2	14
	2	200	38	33	31	34	±	2	17
CuATSM/H_2ATSM (30.00 µg/ml)	1	200	--[b]	--	--	--		--	--
	2	200	--[b]	--	--	--		--	--
CuATSM/H_2ATSM (40.00 µg/ml)	1	200	--[b]	--	--	--		--	--
	2	200	--[b]	--	--	--		--	--
CuATSM/H_2ATSM (100.00 µg/ml)	1	200	--[b]	--	--	--		--	--
	2	200	--[b]	--	--	--		--	--

[a] Cloning Efficiency = (Mean number of colonies in viable count plates ÷ Total number of cells seeded) x 100

[b] -- = Not analyzable due to excessive cytotoxicity

EVALUATION OF THE POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM (NSC-D729307) IN THE MOUSE LYMPHOMA ASSAY

Appendix B Table B-4

Cytotoxicity Assay (Initial Mutagenicity Assay) - with S9

Relative Suspension Growth

Test Article (Concentration)	Sample ID	Day 1 Cell Concentration (cells/ml)	Day 1 %Relative Suspension Growth (RSG)[c]	Day 2 Cell Concentration (cells/ml)	Day 2 %Relative Suspension Growth (RSG)[c]	Cumulative %Relative Suspension Growth[d]
Media Control	1	$9.98x10^5$	98	$1.09 x10^6$	103	101
	2	$1.04x10^6$	102	$1.03 x10^6$	97	99
Vehicle Control (0.0%)	1	$9.56x10^5$	95	$1.13 x10^6$	99	94
	2	$1.06x10^6$	105	$1.15 x10^6$	101	106
Methyl Methanesulfonate (26.8 µg/ml)	1	$6.08x10^5$	60	$9.15 x10^5$	80	48
	2	$5.72x10^5$	57	$8.33 x10^5$	73	42
CuATSM/H_2ATSM (0.25 µg/ml)	1	$1.03x10^6$	102	$1.03x10^6$	90	92
	2	$1.11x10^6$	110	$1.05x10^6$	92	101
CuATSM/H_2ATSM (0.50 µg/ml)	1	$9.42x10^5$	93	$1.16x10^6$	101	94
	2	$1.05x10^6$	104	$1.17x10^6$	103	107
CuATSM/H_2ATSM (1.00 µg/ml)	1	$1.00x10^6$	99	$1.18x10^6$	103	102
	2	$9.40x10^5$	93	$1.18x10^6$	104	97
CuATSM/H_2ATSM (3.00 µg/ml)	1	$9.02x10^5$	89	$1.13x10^6$	99	88
	2	$8.59x10^5$	85	$8.10x10^5$	71	60
CuATSM/H_2ATSM (5.00 µg/ml)	1	$9.40x10^5$	93	$7.84x10^5$	69	64
	2	$9.24x10^5$	91	$1.04x10^6$	91	83
CuATSM/H_2ATSM (10.00 µg/ml)	1	$8.29x10^5$	82	$9.16x10^5$	80	66
	2	$8.07x10^5$	80	$9.64x10^5$	84	67
CuATSM/H_2ATSM (25.00 µg/ml)	1	$2.72x10^5$	27	$3.76x10^5$	33	9
	2	$3.30x10^5$	33	$6.27x10^5$	55	18
CuATSM/H_2ATSM (30.00 µg/ml)	1	$2.08x10^5$	21	--[b]	--	--
	2	$2.16x10^5$	21	--[b]	--	--
CuATSM/H_2ATSM (40.00 µg/ml)	1	$1.72x10^5$	17	--[b]	--	--
	2	$2.68x10^5$	27	--[b]	--	--
CuATSM/H_2ATSM (100.00 µg/ml)	1	$2.02x10^5$	20	--[b]	--	--
	2	$3.60x10^4$	4	--[b]	--	--

[b] -- = Not analyzable due to excessive cytotoxicity

[c] Methyl Methanesulfonate and test articles are relative to the vehicle control

[d] Cumulative %Relative Suspension Growth = [(Day 1 RSG) x (Day 2 RSG)] ÷ 100 (original un-rounded values were used for calculations)

EVALUATION OF THE POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM (NSC-D729307) IN THE MOUSE LYMPHOMA ASSAY

Appendix B Table B-5

Repeat Cytotoxicity Assay (Repeat Initial Mutagenicity Assay) - without S9

Colony Counts per Plate and Cloning Efficiency

Test Article (Concentration)	Sample ID	Cells Seeded	Viable Count Plates A	B	C	Mean	±	SD	Cloning Efficiency (CE)[a]
Media Control	1	200	199	216	197	204	±	6	102
	2	200	149	171	139	153	±	9	76
Vehicle Control (1.0%)	1	200	214	218	202	211	±	5	105
	2	200	204	208	184	199	±	8	99
Methyl Methanesulfonate (26.8 µg/ml)	1	200	149	137	146	144	±	3	72
	2	200	209	208	217	211	±	3	105
CuATSM/H_2ATSM (3.00 µg/ml)	1	200	197	199	213	203	±	5	101
	2	200	223	210	224	219	±	5	109
CuATSM/H_2ATSM (5.00 µg/ml)	1	200	238	231	233	234	±	2	117
	2	200	186	201	197	195	±	5	97
CuATSM/H_2ATSM (7.00 µg/ml)	1	200	209	201	189	200	±	6	100
	2	200	197	185	191	191	±	3	95
CuATSM/H_2ATSM (10.00 µg/ml)	1	200	101	102	108	104	±	2	52
	2	200	125	105	115	115	±	6	57
CuATSM/H_2ATSM (12.00 µg/ml)	1	200	123	99	133	118	±	10	59
	2	200	109	89	125	108	±	10	54
CuATSM/H_2ATSM (15.00 µg/ml)	1	200	102	96	99	99	±	2	49
	2	200	94	89	101	95	±	3	47
CuATSM/H_2ATSM (18.00 µg/ml)	1	200	61	50	58	56	±	3	28
	2	200	67	61	67	65	±	3	33
CuATSM/H_2ATSM (20.00 µg/ml)	1	--[b]	--	--	--	--		--	--
	2	--[b]	--	--	--	--		--	--
CuATSM/H_2ATSM (25.00 µg/ml)	1	--[b]	--	--	--	--		--	--
	2	--[b]	--	--	--	--		--	--

[a] Cloning Efficiency = (Mean number of colonies in viable count plates ÷ Total number of cells seeded) x 100

[b] -- = Not analyzable due to excessive cytotoxicity

EVALUATION OF THE POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM (NSC-D729307) IN THE MOUSE LYMPHOMA ASSAY

Appendix B Table B-6

Repeat Cytotoxicity Assay (Repeat Initial Mutagenicity Assay) - without S9

Relative Suspension Growth

Test Article (Concentration)	Sample ID	Day 1 Cell Concentration (cells/ml)	Day 1 %Relative Suspension Growth (RSG)[c]	Day 2 Cell Concentration (cells/ml)	Day 2 %Relative Suspension Growth (RSG)[c]	Cumulative %Relative Suspension Growth[d]
Media Control	1	$9.59x10^5$	106	$1.28x10^6$	103	109.2
	2	$8.50x10^5$	94	$1.21x10^6$	97	91.2
Vehicle Control (1.0%)	1	$9.08x10^5$	100	$1.31x10^6$	101	101.0
	2	$8.67x10^5$	96	$1.28x10^6$	99	95.0
Methyl Methanesulfonate (26.8 µg/ml)	1	$8.60x10^5$	95	$1.32x10^6$	102	96.9
	2	$7.03x10^5$	78	$1.30x10^6$	100	78.0
CuATSM/H_2ATSM (3.00 µg/ml)	1	$6.97x10^5$	77	$1.12x10^6$	87	67.0
	2	$6.68x10^5$	74	$1.11x10^6$	86	63.6
CuATSM/H_2ATSM (5.00 µg/ml)	1	$5.29x10^5$	59	$1.28x10^6$	99	58.4
	2	$5.96x10^5$	66	$9.16x10^5$	71	46.9
CuATSM/H_2ATSM (7.00 µg/ml)	1	$6.07x10^5$	67	$1.17x10^6$	90	60.3
	2	$7.73x10^5$	85	$1.27x10^6$	98	83.3
CuATSM/H_2ATSM (10.00 µg/ml)	1	$5.58x10^5$	62	$1.05x10^6$	81	50.2
	2	$5.24x10^5$	58	$1.18x10^6$	91	52.8
CuATSM/H_2ATSM (12.00 µg/ml)	1	$4.30x10^5$	47	$6.59x10^5$	51	24.0
	2	$4.56x10^5$	50	$7.71x10^5$	59	29.5
CuATSM/H_2ATSM (15.00 µg/ml)	1	$3.12x10^5$	35	$5.48x10^5$	42	14.7
	2	$3.13x10^5$	35	$5.05x10^5$	39	13.7
CuATSM/H_2ATSM (18.00 µg/ml)	1	$2.90x10^5$	32	$4.70x10^5$	36	11.5
	2	$2.58x10^5$	28	$4.98x10^5$	38	10.6
CuATSM/H_2ATSM (20.00 µg/ml)	1	$2.60x10^5$	29	$5.35x10^5$	41	11.9
	2	$2.72x10^5$	30	$4.78x10^5$	37	11.1
CuATSM/H_2ATSM (25.00 µg/ml)	1	$2.26x10^5$	25	--[b]	--	--
	2	$2.20x10^5$	24	--[b]	--	--

[b] -- = Not analyzable due to excessive cytotoxicity

[c] Methyl Methanesulfonate and test articles are relative to the vehicle control

[d] Cumulative %Relative Suspension Growth = [(Day 1 RSG) x (Day 2 RSG)] ÷ 100 (original un-rounded values were used for calculations)

EVALUATION OF THE POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM (NSC-D729307) IN THE MOUSE LYMPHOMA ASSAY

Appendix B Table B-7

Repeat Cytotoxicity Assay (Repeat Initial Mutagenicity Assay) - without S9

Colony Counts per Mutant Plate and Mutation Frequency (Unsized Colonies)

Test Article (Concentration)	Sample ID	Cells Seeded	Mutant Plates A	B	C	Mean ± SD	Mutation Frequency (x10^6)[e]
Media Control	1	200	83	83	109	92 ± 15	90
	2	200	46	53	64	54 ± 9	71
Vehicle Control (1.0%)	1	200	107	118	96	107 ± 11	101
	2	200	88	74	87	83 ± 8	83
Methyl Methanesulfonate (26.8 µg/ml)	1	200	225	219	235	226 ± 8	314
	2	200	277	261	279	272 ± 10	258
CuATSM/H_2ATSM (3.00 µg/ml)	1	200	110	103	87	100 ± 12	99
	2	200	90	83	103	92 ± 10	84
CuATSM/H_2ATSM (5.00 µg/ml)	1	200	97	83	95	92 ± 8	79
	2	200	83	87	87	86 ± 2	88
CuATSM/H_2ATSM (7.00 µg/ml)	1	200	86	82	91	86 ± 5	86
	2	200	88	98	109	98 ± 11	103
CuATSM/H_2ATSM (10.00 µg/ml)	1	200	76	69	58	68 ± 9	131
	2	200	78	82	79	80 ± 2	139
CuATSM/H_2ATSM (12.00 µg/ml)	1	200	81	79	84	81 ± 3	137
	2	200	71	77	76	75 ± 3	139
CuATSM/H_2ATSM (15.00 µg/ml)	1	200	66	65	67	66 ± 1	133
	2	200	65	65	53	61 ± 7	128
CuATSM/H_2ATSM (18.00 µg/ml)	1	200	47	51	45	48 ± 3	171
	2	200	48	46	48	47 ± 1	145
CuATSM/H_2ATSM (20.00 µg/ml)	1	--[b]	--	--	--	--	--
	2	--[b]	--	--	--	--	--
CuATSM/H_2ATSM (25.00 µg/ml)	1	--[b]	--	--	--	--	--
	2	--[b]	--	--	--	--	--

[b] -- = Not analyzable due to excessive cytotoxicity

[e] Mutant frequency = (Mean number of colonies in mutant count plates ÷ Cloning Efficiency) x 100

EVALUATION OF THE POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM (NSC-D729307) IN THE MOUSE LYMPHOMA ASSAY

Appendix B Table B-8

Repeat Cytotoxicity Assay (Repeat Initial Mutagenicity Assay) - without S9

Colony Counts per Mutant Plate and Mutation Frequency (Large Colonies)

Test Article (Concentration)	Sample ID	Cells Seeded	Mutant Plates A	B	C	Mean	±	SD	Mutation Frequency ($x10^6$)[e]
Media Control	1	200	57	48	76	60	±	14	59
	2	200	38	31	31	33	±	4	43
Vehicle Control (1.0%)	1	200	74	78	81	78	±	4	74
	2	200	58	54	65	59	±	6	59
Methyl Methanesulfonate (26.8 µg/ml)	1	200	121	116	120	119	±	3	165
	2	200	152	133	135	140	±	10	133
CuATSM/H_2ATSM (3.00 µg/ml)	1	200	74	71	42	62	±	18	61
	2	200	62	66	88	72	±	14	66
CuATSM/H_2ATSM (5.00 µg/ml)	1	200	54	63	73	63	±	10	54
	2	200	55	57	61	58	±	3	59
CuATSM/H_2ATSM (7.00 µg/ml)	1	200	67	55	58	60	±	6	60
	2	200	54	62	71	62	±	9	65
CuATSM/H_2ATSM (10.00 µg/ml)	1	200	48	39	52	46	±	7	88
	2	200	44	55	62	54	±	9	94
CuATSM/H_2ATSM (12.00 µg/ml)	1	200	43	53	66	54	±	12	92
	2	200	41	41	50	44	±	5	81
CuATSM/H_2ATSM (15.00 µg/ml)	1	200	36	51	48	45	±	8	91
	2	200	36	45	38	40	±	5	84
CuATSM/H_2ATSM (18.00 µg/ml)	1	200	34	37	30	34	±	4	121
	2	200	31	33	34	33	±	2	102
CuATSM/H_2ATSM (20.00 µg/ml)	1	--[b]	--	--	--	--		--	--
	2	--[b]	--	--	--	--		--	--
CuATSM/H_2ATSM (25.00 µg/ml)	1	--[b]	--	--	--	--		--	--
	2	--[b]	--	--	--	--		--	--

[b] -- = Not analyzable due to excessive cytotoxicity

[e] Mutant frequency = (Mean number of colonies in mutant count plates ÷ Cloning Efficiency) x 100

EVALUATION OF THE POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM (NSC-D729307) IN THE MOUSE LYMPHOMA ASSAY

Appendix B Table B-9

Repeat Cytotoxicity Assay (Repeat Initial Mutagenicity Assay) - without S9

Colony Counts per Mutant Plate and Mutation Frequency (Small Colonies)

Test Article (Concentration)	Sample ID	Cells Seeded	Mutant Plates A	B	C	Mean	±	SD	Mutation Frequency (x10^6)[e]
Media Control	1	200	26	35	33	31	±	5	30
	2	200	8	22	33	21	±	13	27
Vehicle Control (1.0%)	1	200	33	40	15	29	±	13	27
	2	200	30	20	22	24	±	5	24
Methyl Methanesulfonate (26.8 µg/ml)	1	200	104	103	115	107	±	7	149
	2	200	125	128	144	132	±	10	125
CuATSM/H_2ATSM (3.00 µg/ml)	1	200	36	32	45	38	±	7	37
	2	200	28	17	15	20	±	7	18
CuATSM/H_2ATSM (5.00 µg/ml)	1	200	43	20	22	28	±	13	24
	2	200	28	30	26	28	±	2	29
CuATSM/H_2ATSM (7.00 µg/ml)	1	200	19	27	33	26	±	7	26
	2	200	34	36	38	36	±	2	38
CuATSM/H_2ATSM (10.00 µg/ml)	1	200	28	30	6	21	±	13	40
	2	200	34	27	17	26	±	9	45
CuATSM/H_2ATSM (12.00 µg/ml)	1	200	38	26	18	27	±	10	46
	2	200	30	36	26	31	±	5	57
CuATSM/H_2ATSM (15.00 µg/ml)	1	200	30	14	19	21	±	8	42
	2	200	29	20	15	21	±	7	44
CuATSM/H_2ATSM (18.00 µg/ml)	1	200	13	14	15	14	±	1	50
	2	200	17	13	14	15	±	2	46
CuATSM/H_2ATSM (20.00 µg/ml)	1	--[b]	--	--	--	--		--	--
	2	--[b]	--	--	--	--		--	--
CuATSM/H_2ATSM (25.00 µg/ml)	1	--[b]	--	--	--	--		--	--
	2	--[b]	--	--	--	--		--	--

[b] -- = Not analyzable due to excessive cytotoxicity

[e] Mutant frequency = (Mean number of colonies in mutant count plates ÷ Cloning Efficiency) x 100

EVALUATION OF THE POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM (NSC-D729307) IN THE MOUSE LYMPHOMA ASSAY

Appendix B Table B-10

Repeat Cytotoxicity Assay (Repeat Initial Mutagenicity Assay) - with S9

Colony Counts per Plate and Cloning Efficiency

Test Article (Concentration)	Sample ID	Cells Seeded	Viable Count Plates A	B	C	Mean	±	SD	Cloning Efficiency (CE)[a]
Media Control	1	200	196	187	199	194	±	3	97
	2	200	182	181	208	190	±	9	95
Vehicle Control (1.0%)	1	200	253	197	233	228	±	16	114
	2	200	256	231	236	241	±	8	120
Benzo[a]Pyrene (2.0 µg/ml)	1	200	76	79	97	84	±	6	42
	2	200	99	99	91	96	±	3	48
CuATSM/H_2ATSM (5.00 µg/ml)	1	200	220	194	190	201	±	9	100
	2	200	188	208	224	207	±	10	103
CuATSM/H_2ATSM (7.00 µg/ml)	1	200	177	187	218	194	±	12	97
	2	200	187	184	199	190	±	5	95
CuATSM/H_2ATSM (10.00 µg/ml)	1	200	166	155	171	164	±	5	82
	2	200	159	165	182	169	±	7	84
CuATSM/H_2ATSM (12.00 µg/ml)	1	200	173	176	180	176	±	2	88
	2	200	201	213	225	213	±	7	106
CuATSM/H_2ATSM (15.00 µg/ml)	1	200	182	201	171	185	±	9	92
	2	200	174	174	201	183	±	9	91
CuATSM/H_2ATSM (18.00 µg/ml)	1	200	141	148	156	148	±	5	74
	2	200	147	179	149	158	±	10	79
CuATSM/H_2ATSM (20.00 µg/ml)	1	200	122	123	117	121	±	2	60
	2	200	100	88	99	96	±	4	48
CuATSM/H_2ATSM (25.00 µg/ml)	1	--[b]	--	--	--	--		--	--
	2	--[b]	--	--	--	--		--	--

[a] Cloning Efficiency = (Mean number of colonies in viable count plates ÷ Total number of cells seeded) x 100

[b] -- = Not analyzable due to excessive cytotoxicity

EVALUATION OF THE POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM (NSC-D729307) IN THE MOUSE LYMPHOMA ASSAY

Appendix B Table B-11

Repeat Cytotoxicity Assay (Repeat Initial Mutagenicity Assay) - with S9

Relative Suspension Growth

		Day 1		Day 2		Cumulative
Test Article (Concentration)	Sample ID	Cell Concentration (cells/ml)	%Relative Suspension Growth (RSG)[c]	Cell Concentration (cells/ml)	%Relative Suspension Growth (RSG)[c]	%Relative Suspension Growth[d]
Media Control	1	$8.29x10^5$	99	$1.25x10^6$	98	97.0
	2	$8.44x10^5$	101	$1.29x10^6$	102	103.0
Vehicle Control (1.0%)	1	$8.81x10^5$	100	$1.30x10^6$	101	101.0
	2	$8.81x10^5$	100	$1.28x10^6$	99	99.0
Benzo[a]Pyrene (2.0 µg/ml)	1	$6.42x10^5$	73	$1.14x10^6$	88	64.2
	2	$6.55x10^5$	74	$1.15x10^6$	89	65.9
CuATSM/H_2ATSM (5.00 µg/ml)	1	$9.00x10^5$	102	$1.57x10^6$	122	124.4
	2	$8.07x10^5$	92	$1.55x10^6$	120	110.4
CuATSM/H_2ATSM (7.00 µg/ml)	1	$8.26x10^5$	94	$1.38x10^6$	107	100.6
	2	$8.45x10^5$	96	$1.92x10^6$	149	143.0
CuATSM/H_2ATSM (10.00 µg/ml)	1	$7.60x10^5$	86	$1.81x10^6$	140	120.4
	2	$8.18x10^5$	93	$1.96x10^6$	152	141.4
CuATSM/H_2ATSM (12.00 µg/ml)	1	$6.04x10^5$	69	$1.70x10^6$	132	91.1
	2	$6.82x10^5$	77	$1.52x10^6$	118	90.9
CuATSM/H_2ATSM (15.00 µg/ml)	1	$6.73x10^5$	76	$1.33x10^6$	103	78.3
	2	$7.17x10^5$	81	$8.01x10^5$	62	50.2
CuATSM/H_2ATSM (18.00 µg/ml)	1	$6.08x10^5$	69	$1.06x10^6$	83	57.3
	2	$4.29x10^5$	49	$9.08x10^5$	70	34.3
CuATSM/H_2ATSM (20.00 µg/ml)	1	$3.63x10^5$	41	$5.65x10^5$	45	18.5
	2	$3.35x10^5$	38	$5.47x10^5$	43	16.3
CuATSM/H_2ATSM (25.00 µg/ml)	1	$2.48x10^5$	28	$3.48x10^5$	27	7.6
	2	$2.36x10^5$	27	$3.62x10^5$	29	7.8

[c] Benzo[a]Pyrene and test articles are relative to the vehicle control

[d] Cumulative %Relative Suspension Growth = [(Day 1 RSG) x (Day 2 RSG)] ÷ 100 (original un-rounded values were used for calculations)

EVALUATION OF THE POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM (NSC-D729307) IN THE MOUSE LYMPHOMA ASSAY

Appendix B Table B-12

Repeat Cytotoxicity Assay (Repeat Initial Mutagenicity Assay) - with S9

Colony Counts per Mutant Plate and Mutation Frequency (Unsized Colonies)

Test Article (Concentration)	Sample ID	Cells Seeded	Mutant Plates A	B	C	Mean	±	SD	Mutation Frequency ($x10^6$)[e]
Media Control	1	200	69	64	68	67	±	3	69
	2	200	71	78	91	80	±	10	84
Vehicle Control (1.0%)	1	200	86	101	104	97	±	10	85
	2	200	110	104	107	107	±	3	89
Benzo[a]Pyrene (2.0 µg/ml)	1	200	222	235	238	232	±	9	552
	2	200	245	252	234	244	±	9	508
CuATSM/H_2ATSM (5.00 µg/ml)	1	200	85	84	97	89	±	7	89
	2	200	82	69	96	82	±	14	79
CuATSM/H_2ATSM (7.00 µg/ml)	1	200	91	99	96	95	±	4	98
	2	200	79	76	94	83	±	10	87
CuATSM/H_2ATSM (10.00 µg/ml)	1	200	63	56	78	66	±	11	81
	2	200	90	77	81	83	±	7	98
CuATSM/H_2ATSM (12.00 µg/ml)	1	200	79	98	77	85	±	12	97
	2	200	77	81	89	82	±	6	77
CuATSM/H_2ATSM (15.00 µg/ml)	1	200	85	109	82	92	±	15	99
	2	200	78	79	91	83	±	7	91
CuATSM/H_2ATSM (18.00 µg/ml)	1	200	58	67	65	63	±	5	85
	2	200	80	84	92	85	±	6	108
CuATSM/H_2ATSM (20.00 µg/ml)	1	200	94	66	81	80	±	14	132
	2	200	72	77	69	73	±	4	152
CuATSM/H_2ATSM (25.00 µg/ml)	1	--[b]	--	--	--	--		--	--
	2	--[b]	--	--	--	--		--	--

[b] -- = Not analyzable due to excessive cytotoxicity

[e] Mutant frequency = (Mean number of colonies in mutant count plates ÷ Cloning Efficiency) x 100

EVALUATION OF THE POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM (NSC-D729307) IN THE MOUSE LYMPHOMA ASSAY

Appendix B Table B-13

Repeat Cytotoxicity Assay (Repeat Initial Mutagenicity Assay) - with S9

Colony Counts per Mutant Plate and Mutation Frequency (Large Colonies)

Test Article (Concentration)	Sample ID	Cells Seeded	Mutant Plates A	B	C	Mean	±	SD	Mutation Frequency (x10^6)[e]
Media Control	1	200	41	39	46	42	±	4	43
	2	200	48	54	54	52	±	3	55
Vehicle Control (1.0%)	1	200	50	60	80	63	±	15	55
	2	200	72	68	75	72	±	4	60
Benzo[a]Pyrene (2.0 µg/ml)	1	200	116	106	131	118	±	13	281
	2	200	112	110	120	114	±	5	238
CuATSM/H_2ATSM (5.00 µg/ml)	1	200	57	53	62	57	±	5	57
	2	200	52	54	71	59	±	10	57
CuATSM/H_2ATSM (7.00 µg/ml)	1	200	63	66	64	64	±	2	66
	2	200	45	39	58	47	±	10	49
CuATSM/H_2ATSM (10.00 µg/ml)	1	200	41	33	41	38	±	5	46
	2	200	54	41	43	46	±	7	54
CuATSM/H_2ATSM (12.00 µg/ml)	1	200	51	60	45	52	±	8	59
	2	200	37	53	45	45	±	8	42
CuATSM/H_2ATSM (15.00 µg/ml)	1	200	55	71	57	61	±	9	66
	2	200	48	44	60	51	±	8	56
CuATSM/H_2ATSM (18.00 µg/ml)	1	200	35	40	66	47	±	17	64
	2	200	52	38	64	51	±	13	65
CuATSM/H_2ATSM (20.00 µg/ml)	1	200	49	31	44	41	±	9	68
	2	200	54	46	31	44	±	12	92
CuATSM/H_2ATSM (25.00 µg/ml)	1	--[b]	--	--	--	--		--	--
	2	--[b]	--	--	--	--		--	--

[b] -- = Not analyzable due to excessive cytotoxicity

[e] Mutant frequency = (Mean number of colonies in mutant count plates ÷ Cloning Efficiency) x 100

EVALUATION OF THE POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM (NSC-D729307) IN THE MOUSE LYMPHOMA ASSAY

Appendix B Table B-14

Repeat Cytotoxicity Assay (Repeat Initial Mutagenicity Assay) - with S9

Colony Counts per Mutant Plate and Mutation Frequency (Small Colonies)

Test Article (Concentration)	Sample ID	Cells Seeded	Mutant Plates A	B	C	Mean	±	SD	Mutation Frequency (x10^6)[e]
Media Control	1	200	28	25	22	25	±	3	26
	2	200	23	24	37	28	±	8	29
Vehicle Control (1.0%)	1	200	36	41	24	34	±	9	30
	2	200	38	36	32	35	±	3	29
Benzo[a]Pyrene (2.0 µg/ml)	1	200	106	129	107	114	±	13	271
	2	200	133	142	114	130	±	14	271
CuATSM/H_2ATSM (5.00 µg/ml)	1	200	28	31	35	31	±	4	31
	2	200	30	15	25	23	±	8	22
CuATSM/H_2ATSM (7.00 µg/ml)	1	200	28	33	32	31	±	3	32
	2	200	34	37	36	36	±	2	38
CuATSM/H_2ATSM (10.00 µg/ml)	1	200	22	23	37	27	±	8	33
	2	200	36	36	38	37	±	1	44
CuATSM/H_2ATSM (12.00 µg/ml)	1	200	28	38	32	33	±	5	38
	2	200	40	28	44	37	±	8	35
CuATSM/H_2ATSM (15.00 µg/ml)	1	200	30	38	25	31	±	7	34
	2	200	30	35	--[b]	33	±	4	36
CuATSM/H_2ATSM (18.00 µg/ml)	1	200	23	27	0	16	±	15	22
	2	200	28	46	28	34	±	10	43
CuATSM/H_2ATSM (20.00 µg/ml)	1	200	45	35	37	39	±	5	64
	2	200	18	31	38	29	±	10	60
CuATSM/H_2ATSM (25.00 µg/ml)	1	--[b]	--	--	--	--		--	--
	2	--[b]	--	--	--	--		--	--

[b] -- = Not analyzable due to excessive cytotoxicity

[e] Mutant frequency = (Mean number of colonies in mutant count plates ÷ Cloning Efficiency) x 100

EVALUATION OF THE POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM (NSC-D729307) IN THE MOUSE LYMPHOMA ASSAY

Appendix B Table B-15

Mutagenicity Assay (Confirmatory Mutagenicity Assay) - without S9

Colony Counts per Plate and Cloning Efficiency

Test Article (Concentration)	Sample ID	Cells Seeded	Viable Count Plates A	B	C	Mean	±	SD	Cloning Efficiency (CE)[a]
Media Control	1	200	162	144	187	164	±	13	82
	2	200	192	167	151	170	±	12	85
Vehicle Control (1.0%)	1	200	139	154	145	146	±	5	73
	2	200	147	127	150	141	±	8	70
Methyl Methanesulfonate (26.8 µg/ml)	1	200	59	65	58	61	±	2	30
	2	200	79	68	71	73	±	3	36
CuATSM/H_2ATSM (0.50 µg/ml)	1	200	101	101	88	97	±	5	48
	2	200	89	89	70	83	±	6	41
CuATSM/H_2ATSM (1.00 µg/ml)	1	200	121	109	92	107	±	9	53
	2	200	102	92	95	96	±	3	48
CuATSM/H_2ATSM (3.00 µg/ml)	1	200	61	59	77	66	±	6	33
	2	200	65	48	66	60	±	6	30
CuATSM/H_2ATSM (5.00 µg/ml)	1	200	49	52	43	48	±	3	24
	2	200	53	51	69	58	±	6	29
CuATSM/H_2ATSM (10.00 µg/ml)	1	172	--[b]	--	--	--		--	--
	2	200	--[b]	--	--	--		--	--
CuATSM/H_2ATSM (12.00 µg/ml)	1	200	--[b]	--	--	--		--	--
	2	200	--[b]	--	--	--		--	--
CuATSM/H_2ATSM (15.00 µg/ml)	1	200	--[b]	--	--	--		--	--
	2	200	--[b]	--	--	--		--	--

[a] Cloning Efficiency = (Mean number of colonies in viable count plates ÷ Total number of cells seeded) x 100

[b] -- = Not analyzable due to excessive cytotoxicity

EVALUATION OF THE POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM (NSC-D729307) IN THE MOUSE LYMPHOMA ASSAY

Appendix B Table B-16

Mutagenicity Assay (Confirmatory Mutagenicity Assay) - without S9

Relative Suspension Growth

Test Article (Concentration)	Sample ID	Day 1 Cell Concentration (cells/ml)	Day 1 %Relative Suspension Growth (RSG)[c]	Day 2 Cell Concentration (cells/ml)	Day 2 %Relative Suspension Growth (RSG)[c]	Cumulative %Relative Suspension Growth[d]
Media Control	1	$8.90x10^5$	96	$1.09x10^6$	91	87.4
	2	$9.72x10^5$	104	$1.32x10^6$	109	113.4
Vehicle Control (1.0%)	1	$7.06x10^5$	76	$9.85x10^5$	108	82.1
	2	$7.22x10^5$	78	$8.47x10^5$	92	71.8
Methyl Methanesulfonate (26.8 µg/ml)	1	$5.81x10^5$	62	$6.79x10^5$	74	45.9
	2	$6.63x10^5$	71	$6.42x10^5$	70	49.7
CuATSM/H_2ATSM (0.50 µg/ml)	1	$6.70x10^5$	72	$9.83x10^5$	107	77.0
	2	$6.93x10^5$	74	$1.03x10^6$	113	83.6
CuATSM/H_2ATSM (1.00 µg/ml)	1	$7.65x10^5$	82	$9.52x10^5$	104	85.3
	2	$6.81x10^5$	73	$1.00x10^6$	109	79.6
CuATSM/H_2ATSM (3.00 µg/ml)	1	$5.39x10^5$	58	$6.39x10^5$	70	40.6
	2	$4.42x10^5$	47	$6.74x10^5$	74	34.8
CuATSM/H_2ATSM (5.00 µg/ml)	1	$4.20x10^5$	45	$6.00x10^5$	66	29.7
	2	$4.82x10^5$	52	$6.09x10^5$	66	34.3
CuATSM/H_2ATSM (10.00 µg/ml)	1	$1.98x10^5$	21	$2.58x10^5$	28	5.9
	2	$2.16x10^5$	23	$3.15x10^5$	34	7.8
CuATSM/H_2ATSM (12.00 µg/ml)	1	$1.51x10^5$	16	--[b]	--	--
	2	$1.43x10^5$	15	--[b]	--	--
CuATSM/H_2ATSM (15.00 µg/ml)	1	$8.33x10^4$	9	--[b]	--	--
	2	$9.39x10^4$	13	--[b]	--	--

[b] -- = Not analyzable due to excessive cytotoxicity

[c] Methyl Methanesulfonate and test articles are relative to the vehicle control

[d] Cumulative %Relative Suspension Growth = [(Day 1 RSG) x (Day 2 RSG)] ÷ 100 (original un-rounded values were used for calculations)

EVALUATION OF THE POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM (NSC-D729307) IN THE MOUSE LYMPHOMA ASSAY

Appendix B Table B-17

Mutagenicity Assay (Confirmatory Mutagenicity Assay) - without S9

Colony Counts per Mutant Plate and Mutation Frequency (Unsized Colonies)

Test Article (Concentration)	Sample ID	Cells Seeded	Mutant Plates A	B	C	Mean	±	SD	Mutation Frequency (x10^6)[e]
Media Control	1	200	50	73	82	68	±	17	83
	2	200	66	61	60	62	±	3	73
Vehicle Control (1.0%)	1	200	41	35	40	39	±	3	53
	2	200	54	67	69	63	±	8	89
Methyl Methanesulfonate (26.8 µg/ml)	1	200	150	152	162	155	±	6	508
	2	200	171	166	157	165	±	7	452
CuATSM/H_2ATSM (0.50 µg/ml)	1	200	24	20	28	24	±	4	49
	2	200	43	39	48	43	±	5	104
CuATSM/H_2ATSM (1.00 µg/ml)	1	200	50	42	32	41	±	9	77
	2	200	32	24	35	30	±	6	63
CuATSM/H_2ATSM (3.00 µg/ml)	1	200	21	18	22	20	±	2	61
	2	200	31	30	30	30	±	1	100
CuATSM/H_2ATSM (5.00 µg/ml)	1	200	33	24	28	28	±	5	117
	2	200	26	22	25	24	±	2	83
CuATSM/H_2ATSM (10.00 µg/ml)	1	172	--[b]	--	--	--		--	--
	2	200	--[b]	--	--	--		--	--
CuATSM/H_2ATSM (12.00 µg/ml)	1	200	--[b]	--	--	--		--	--
	2	200	--[b]	--	--	--		--	--
CuATSM/H_2ATSM (15.00 µg/ml)	1	200	--[b]	--	--	--		--	--
	2	200	--[b]	--	--	--		--	--

[b] -- = Not analyzable due to excessive cytotoxicity

[e] Mutant frequency = (Mean number of colonies in mutant count plates ÷ Cloning Efficiency) x 100

EVALUATION OF THE POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM (NSC-D729307) IN THE MOUSE LYMPHOMA ASSAY

Appendix B Table B-18

Mutagenicity Assay (Confirmatory Mutagenicity Assay) - without S9

Colony Counts per Mutant Plate and Mutation Frequency (Large Colonies)

Test Article (Concentration)	Sample ID	Cells Seeded	Mutant Plates A	B	C	Mean ± SD	Mutation Frequency ($x10^6$)[e]
Media Control	1	200	32	47	52	44 ± 10	54
	2	200	47	34	26	36 ± 11	42
Vehicle Control (1.0%)	1	200	21	24	26	24 ± 3	33
	2	200	37	33	32	34 ± 3	48
Methyl Methanesulfonate (26.8 µg/ml)	1	200	73	66	76	72 ± 5	236
	2	200	83	83	65	77 ± 10	211
CuATSM/H_2ATSM (0.50 µg/ml)	1	200	14	13	10	12 ± 2	25
	2	200	21	19	24	21 ± 3	51
CuATSM/H_2ATSM (1.00 µg/ml)	1	200	33	21	18	24 ± 8	45
	2	200	20	14	25	20 ± 6	42
CuATSM/H_2ATSM (3.00 µg/ml)	1	200	12	11	12	12 ± 1	36
	2	200	25	23	18	22 ± 4	73
CuATSM/H_2ATSM (5.00 µg/ml)	1	200	23	14	17	18 ± 5	75
	2	200	16	8	17	14 ± 5	48
CuATSM/H_2ATSM (10.00 µg/ml)	1	172	--[b]	--	--	--	--
	2	200	--[b]	--	--	--	--
CuATSM/H_2ATSM (12.00 µg/ml)	1	200	--[b]	--	--	--	--
	2	200	--[b]	--	--	--	--
CuATSM/H_2ATSM (15.00 µg/ml)	1	200	--[b]	--	--	--	--
	2	200	--[b]	--	--	--	--

[b] -- = Not analyzable due to excessive cytotoxicity

[e] Mutant frequency = (Mean number of colonies in mutant count plates ÷ Cloning Efficiency) x 100

EVALUATION OF THE POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM (NSC-D729307) IN THE MOUSE LYMPHOMA ASSAY

Appendix B Table B-19

Mutagenicity Assay (Confirmatory Mutagenicity Assay) - without S9

Colony Counts per Mutant Plate and Mutation Frequency (Small Colonies)

Test Article (Concentration)	Sample ID	Cells Seeded	Mutant Plates A	B	C	Mean	±	SD	Mutation Frequency ($x10^6$)[e]
Media Control	1	200	18	26	30	25	±	6	30
	2	200	19	27	34	27	±	8	32
Vehicle Control (1.0%)	1	200	20	11	14	15	±	5	21
	2	200	17	34	37	29	±	11	41
Methyl Methanesulfonate (26.8 µg/ml)	1	200	77	86	86	83	±	5	272
	2	200	88	83	92	88	±	5	241
CuATSM/H_2ATSM (0.50 µg/ml)	1	200	10	7	18	12	±	6	25
	2	200	22	20	24	22	±	2	53
CuATSM/H_2ATSM (1.00 µg/ml)	1	200	17	21	14	17	±	4	32
	2	200	12	10	10	11	±	1	23
CuATSM/H_2ATSM (3.00 µg/ml)	1	200	9	7	10	9	±	2	27
	2	200	6	7	12	8	±	3	27
CuATSM/H_2ATSM (5.00 µg/ml)	1	200	10	10	11	10	±	1	42
	2	200	10	14	8	11	±	3	38
CuATSM/H_2ATSM (10.00 µg/ml)	1	172	--[b]	--	--	--		--	--
	2	200	--[b]	--	--	--		--	--
CuATSM/H_2ATSM (12.00 µg/ml)	1	200	--[b]	--	--	--		--	--
	2	200	--[b]	--	--	--		--	--
CuATSM/H_2ATSM (15.00 µg/ml)	1	200	--[b]	--	--	--		--	--
	2	200	--[b]	--	--	--		--	--

[b] -- = Not analyzable due to excessive cytotoxicity

[c] Mutant frequency = (Mean number of colonies in mutant count plates ÷ Cloning Efficiency) x 100

EVALUATION OF THE POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM (NSC-D729307) IN THE MOUSE LYMPHOMA ASSAY

Appendix B Table B-20

Mutagenicity Assay (Confirmatory Mutagenicity Assay) - with S9

Colony Counts per Plate and Cloning Efficiency

Test Article (Concentration)	Sample ID	Cells Seeded	Viable Count Plates A	B	C	Mean	±	SD	Cloning Efficiency (CE)[a]
Media Control	1	200	140	165	194	166	±	16	83
	2	200	165	149	140	151	±	8	75
Vehicle Control (1.0%)	1	200	167	194	205	189	±	12	94
	2	200	211	178	216	202	±	12	101
Benzo[a]Pyrene (2.0 µg/ml)	1	200	135	105	103	114	±	10	57
	2	200	54	57	86	66	±	10	33
CuATSM/H_2ATSM (10.00 µg/ml)	1	200	124	135	116	125	±	6	62
	2	200	200	167	170	179	±	10	89
CuATSM/H_2ATSM (12.00 µg/ml)	1	200	130	123	107	120	±	7	60
	2	200	100	100	103	101	±	1	50
CuATSM/H_2ATSM (15.00 µg/ml)	1	200	176	211	173	187	±	12	93
	2	200	230	232	227	230	±	2	115
CuATSM/H_2ATSM (18.00 µg/ml)	1	200	132	124	108	121	±	7	60
	2	200	178	197	192	189	±	6	94
CuATSM/H_2ATSM (20.00 µg/ml)	1	200	108	98	105	104	±	3	52
	2	200	130	140	127	132	±	4	66

[a] Cloning Efficiency = (Mean number of colonies in viable count plates ÷ Total number of cells seeded) x 100

EVALUATION OF THE POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM (NSC-D729307) IN THE MOUSE LYMPHOMA ASSAY

Appendix B Table B-21

Mutagenicity Assay (Confirmatory Mutagenicity Assay) - with S9

Relative Suspension Growth

		Day 1		Day 2		Cumulative
Test Article (Concentration)	Sample ID	Cell Concentration (cells/ml)	%Relative Suspension Growth (RSG)[c]	Cell Concentration (cells/ml)	%Relative Suspension Growth (RSG)[c]	%Relative Suspension Growth[d]
Media Control	1	$7.49x10^5$	97	$9.28x10^5$	95	92.2
	2	$8.02x10^5$	103	$1.02x10^6$	105	108.2
Vehicle Control (1.0%)	1	$6.67x10^5$	96	$9.98x10^5$	98	94.1
	2	$7.27x10^5$	104	$1.03x10^6$	102	106.1
Benzo[a]Pyrene (2.0 µg/ml)	1	$4.10x10^5$	59	$7.76x10^5$	76	44.8
	2	$4.22x10^5$	60	$7.33x10^5$	72	43.2
CuATSM/H_2ATSM (10.00 µg/ml)	1	$6.99x10^5$	100	$1.18x10^6$	117	117.0
	2	$6.05x10^5$	87	$1.04x10^6$	103	89.6
CuATSM/H_2ATSM (12.00 µg/ml)	1	$7.13x10^5$	102	$1.06x10^6$	104	106.1
	2	$6.77x10^5$	97	$9.95x10^5$	98	95.1
CuATSM/H_2ATSM (15.00 µg/ml)	1	$6.11x10^5$	88	$9.94x10^5$	98	86.2
	2	$6.67x10^5$	96	$9.25x10^5$	91	87.4
CuATSM/H_2ATSM (18.00 µg/ml)	1	$5.26x10^5$	75	$6.73x10^5$	66	49.5
	2	$5.64x10^5$	81	$7.37x10^5$	73	59.1
CuATSM/H_2ATSM (20.00 µg/ml)	1	$4.00x10^5$	57	$7.82x10^5$	77	43.9
	2	$4.69x10^5$	67	$7.48x10^5$	74	49.6

[c] Benzo[a]Pyrene and test articles are relative to the vehicle control

[d] Cumulative %Relative Suspension Growth = [(Day 1 RSG) x (Day 2 RSG)] ÷ 100 (original un-rounded values were used for calculations)

EVALUATION OF THE POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM (NSC-D729307) IN THE MOUSE LYMPHOMA ASSAY

Appendix B Table B-22

Mutagenicity Assay (Confirmatory Mutagenicity Assay) - with S9

Colony Counts per Mutant Plate and Mutation Frequency (Unsized Colonies)

Test Article (Concentration)	Sample ID	Cells Seeded	Mutant Plates A	B	C	Mean	±	SD	Mutation Frequency (x10^6)[e]
Media Control	1	200	70	76	103	83	±	18	100
	2	200	49	62	68	60	±	10	79
Vehicle Control (1.0%)	1	200	86	59	124	90	±	33	95
	2	200	73	78	111	87	±	21	86
Benzo[a]Pyrene (2.0 µg/ml)	1	200	489	451	435	458	±	28	804
	2	200	308	275	203	262	±	54	794
CuATSM/H_2ATSM (10.00 µg/ml)	1	200	89	73	65	76	±	12	122
	2	200	89	71	73	78	±	10	87
CuATSM/H_2ATSM (12.00 µg/ml)	1	200	71	66	82	73	±	8	122
	2	200	64	66	81	70	±	9	139
CuATSM/H_2ATSM (15.00 µg/ml)	1	200	78	81	89	83	±	6	89
	2	200	68	108	97	91	±	21	79
CuATSM/H_2ATSM (18.00 µg/ml)	1	200	97	65	54	72	±	22	119
	2	200	116	95	138	116	±	22	123
CuATSM/H_2ATSM (20.00 µg/ml)	1	200	96	76	89	87	±	10	167
	2	200	84	84	75	81	±	5	123

[e] Mutant frequency = (Mean number of colonies in mutant count plates ÷ Cloning Efficiency) x 100

EVALUATION OF THE POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM (NSC-D729307) IN THE MOUSE LYMPHOMA ASSAY

Appendix B Table B-23

Mutagenicity Assay (Confirmatory Mutagenicity Assay) - with S9

Colony Counts per Mutant Plate and Mutation Frequency (Large Colonies)

Test Article (Concentration)	Sample ID	Cells Seeded	Mutant Plates A	B	C	Mean	±	SD	Mutation Frequency ($x10^6$)[e]
Media Control	1	200	54	47	55	52	±	4	63
	2	200	31	38	35	35	±	4	46
Vehicle Control (1.0%)	1	200	55	56	55	55	±	1	58
	2	200	47	52	53	51	±	3	51
Benzo[a]Pyrene (2.0 µg/ml)	1	200	262	307	265	278	±	25	488
	2	200	171	168	132	157	±	22	476
CuATSM/H_2ATSM (10.00 µg/ml)	1	200	61	57	58	59	±	2	94
	2	200	64	54	44	54	±	10	60
CuATSM/H_2ATSM (12.00 µg/ml)	1	200	48	33	59	47	±	13	78
	2	200	37	38	41	39	±	2	77
CuATSM/H_2ATSM (15.00 µg/ml)	1	200	50	51	49	50	±	1	53
	2	200	41	55	40	45	±	8	39
CuATSM/H_2ATSM (18.00 µg/ml)	1	200	57	46	37	47	±	10	78
	2	200	72	71	85	76	±	8	80
CuATSM/H_2ATSM (20.00 µg/ml)	1	200	53	43	56	51	±	7	98
	2	200	61	61	44	55	±	10	83

[e] Mutant frequency = (Mean number of colonies in mutant count plates ÷ Cloning Efficiency) x 100

EVALUATION OF THE POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM (NSC-D729307) IN THE MOUSE LYMPHOMA ASSAY

Appendix B Table B-24

Mutagenicity Assay (Confirmatory Mutagenicity Assay) - with S9

Colony Counts per Mutant Plate and Mutation Frequency (Small Colonies)

Test Article (Concentration)	Sample ID	Cells Seeded	Mutant Plates A	B	C	Mean ± SD	Mutation Frequency ($x10^6$)[e]
Media Control	1	200	16	29	48	31 ± 16	37
	2	200	18	24	33	25 ± 8	33
Vehicle Control (1.0%)	1	200	31	3	69	34 ± 33	36
	2	200	26	26	58	37 ± 18	37
Benzo[a]Pyrene (2.0 µg/ml)	1	200	227	144	170	180 ± 42	316
	2	200	137	107	71	105 ± 33	318
CuATSM/H_2ATSM (10.00 µg/ml)	1	200	28	16	7	17 ± 11	27
	2	200	25	17	29	24 ± 6	27
CuATSM/H_2ATSM (12.00 µg/ml)	1	200	23	33	23	26 ± 6	43
	2	200	27	28	40	32 ± 7	63
CuATSM/H_2ATSM (15.00 µg/ml)	1	200	28	30	40	33 ± 6	35
	2	200	27	53	57	46 ± 16	40
CuATSM/H_2ATSM (18.00 µg/ml)	1	200	40	19	17	25 ± 13	41
	2	200	44	24	53	40 ± 15	42
CuATSM/H_2ATSM (20.00 µg/ml)	1	200	43	33	33	36 ± 6	69
	2	200	23	23	31	26 ± 5	39

[e] Mutant frequency = (Mean number of colonies in mutant count plates ÷ Cloning Efficiency) x 100

Appendix C: Certificates of Analysis

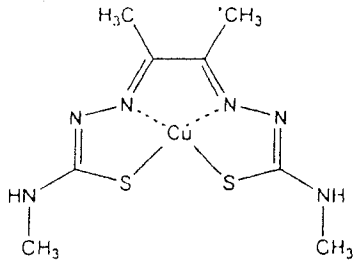
EVALUATION OF THE POTENTIAL MUTAGENIC ACTIVITY OF CuATSM/H_2ATSM (NSC-D729307) IN THE MOUSE LYMPHOMA ASSAY

Appendix C

6336-151
6336-152

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

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

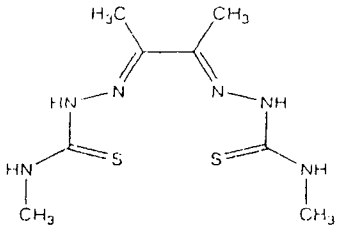
ESI +ve: A 1 mg/ml solution of Cu-ATSM (M) was made up by dissolving 1 mg of material into 1 mL of ethanol. Of this freshly prepared solution 20 µL was removed and added to 200 µL of a 1:1 mixture of water and methanol. This was then directly infused at a flow rate of 10 µL/min into the water ZQ 4000 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.

COPY

6336-151
6336-152

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

H_2ATSM



Elemental Analysis;

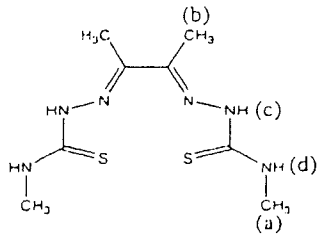
	C	H	N
Expected; Calc for $C_8H_{16}S_2N_6.¼H_2O$	36.40	6.30	31.83
Found (JSL 3697)	36.48	6.23	31.33
Found (JSL 3697 (b))	36.59	6.15	31.77

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