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FINAL REPORT

L5178Y TK ⁺/₋ MOUSE LYMPHOMA FORWARD MUTATION ASSAY
WITH A CONFIRMATORY ASSAY
WITH N-EtFOSE T-6316

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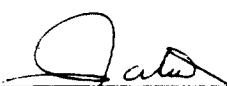
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QUALITY ASSURANCE STATEMENT

L5178Y TK ⁺/₋ Mouse Lymphoma Forward Mutation Assay with a Confirmatory Assay
with N-EtFOSE T-6316

The report has been reviewed by the Quality Assurance Unit of Covance Laboratories Inc., in accordance with the Good Laboratory Practice regulations as set forth in the Food and Drug Administration (FDA) Title 21 of the U.S. Code of Federal Regulations Part 58, and with any applicable amendments. The following inspections were conducted and the findings reported to the Study Director and study director management. Written status reports of inspections and findings are issued to Covance management according to standard operating procedures.

Inspection Dates	Phase	Dates Reported to Study Director and Study Director Management	Auditor
May 31, 2000	Scoring	May 31, 2000	S. Ballinger
July 19-20, 2000	Draft Report Review	July 20, 2000	P. Cáceres
August 30, 2000	Final Report Review	August 30, 2000	P. Cáceres



Representative, Quality Assurance Unit

9/5/00

Date

STUDY COMPLIANCE AND CERTIFICATION

The study was conducted in compliance with the Good Laboratory Practice regulations as set forth in the Food and Drug Administration (FDA) Title 21 of the U.S. Code of Federal Regulations Part 58, and with any applicable amendments. There were no deviations from the regulations or the signed protocol that would affect the integrity of the study or the interpretation of the test results. The raw data have been reviewed by the Study Director, who certifies that the evaluation of the test article as presented herein represents an appropriate conclusion within the context of the study design and evaluation criteria. All test and control results in this report are supported by an experimental data record and this record has been reviewed by the Study Director.

Study Director:

Maria A. Cifone
Maria A. Cifone, PhD
Genetic and Molecular Toxicology

9-5-00
Date

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ABSTRACT

The objective of this *in vitro* assay was to evaluate the ability of the test article, N-EtFOSE T-6316, to induce forward mutations at the thymidine kinase (TK) locus in the mouse lymphoma L5178Y cell line. The test article was soluble in the vehicle (dimethylsulfoxide, DMSO) at 125 mg/mL, the highest concentration prepared.

A dose rangefinding assay was performed with and without metabolic activation using a treatment period of approximately 4 hours. In addition, a dose rangefinding assay without metabolic activation using a treatment period of approximately 24 hours was performed. The dose rangefinding assays were initiated with concentrations from 2.47 to 1250 µg/mL. In the presence and absence of rat liver S9 metabolic activity with a 4-hour treatment period, N-EtFOSE T-6316 was noncytotoxic up to 19.7 µg/mL. Moderate cytotoxicity was induced from 39.3 to 157 µg/mL and higher concentrations were excessively cytotoxic or lethal. In the nonactivation dose rangefinding assay using a 24-hour treatment period, the test article induced no cytotoxicity at 2.47 µg/mL, weak cytotoxicity at 4.93 µg/mL, and moderately high cytotoxicity at 9.85 µg/mL. Higher concentrations were excessively cytotoxic or lethal. Dose levels for the mutation assays were based on these results.

In the nonactivation mutation assay with a treatment period of approximately 4 hours, six doses ranging from 25.3 to 201 µg/mL were analyzed for mutant induction and weak cytotoxicity to high cytotoxicity was induced. None of the analyzed treatments induced a mutant frequency that exceeded the minimum criteria for a positive response. A confirmatory assay was performed.

In the confirmatory nonactivation mutation assay, which was performed with a 24-hour treatment period, seven treatments from 1.25 to 15.0 µg/mL were analyzed and no cytotoxicity to high cytotoxicity was induced. None of the treatments induced a mutant frequency that exceeded the minimum criteria for a positive response. The test article was, therefore, evaluated as negative without metabolic activation.

In the initial mutation assay in the presence of S9 metabolic activation, four treatments from 25.3 to 101 µg/mL were analyzed and weak cytotoxicity to very high cytotoxicity was induced. None of the analyzed treatments in either assay induced a mutant frequency that exceeded the minimum criteria for a positive response. A confirmatory mutation assay was performed.

In the confirmatory activation mutation assay, eight treatments from 20.0 to 150 µg/mL were analyzed and weak cytotoxicity to high cytotoxicity was induced. None of the treatments induced a mutant frequency that exceeded the minimum criteria for a positive response. The test article was, therefore, evaluated as negative with metabolic activation.

The test article, N-EtFOSE T-6316, was evaluated as negative for inducing forward mutations at the TK locus in L5178Y mouse lymphoma cells under activation and nonactivation.

STUDY INFORMATION

Sponsor

3M Corporate Toxicology

Test Article

Sponsor Identification: N-EtFOSE

T-6316

Lot 30035, 30037, 30039

Purity 98.1% (August 19 sample)

TM #: 1084A3 (August 19 sample)

Dates Received: August 19, 1999 and June 07, 2000

Physical Description: white to beige solid (August 19 sample)
beige chunky solid (June 07 sample)

Storage Conditions: room temperature

Assay Information

Type of Assay: L5178Y TK⁺ Mouse Lymphoma Forward Mutation Assay
With A Confirmatory Assay

Protocol No.: 431 ICH Edition 2

Covance Study No.: 20785-0-431 ICH

Study Dates

Initiation Date: May 08, 2000

Experimental Start Date: May 10, 2000

Experimental Termination Date: June 22, 2000

Supervisory Personnel

Study Director: Maria A. Cifone, PhD

Laboratory Supervisor: Kathryn Flanders, MS

OBJECTIVE

The objective of this study was to evaluate the ability of the test article to induce forward mutations at the thymidine kinase (TK) locus of L5178Y mouse lymphoma cells as assayed by colony growth in the presence of 5-trifluorothymidine (TFT). The assay design is based on OECD Guideline 476, updated and adopted July 21, 1997, and the ICH S2B Guideline, "Genotoxicity: Standard Battery for Genotoxicity Testing of Pharmaceuticals".

TEST SYSTEM RATIONALE

Thymidine kinase (TK) is an enzyme that allows cells to salvage thymidine from the culture medium for DNA synthesis. If the thymidine analog TFT is included in the growth medium, the analog will be phosphorylated via the TK pathway and will cause cell death by inhibiting DNA synthesis. Cells which are heterozygous at the TK locus ($TK^{+/-}$) may undergo a single-step forward mutation to the $TK^{-/-}$ genotype in which little or no TK activity remains. Such mutants are viable in normal culture medium because *de novo* DNA synthesis does not require exogenous thymidine. $TK^{-/-}$ mutants, however, are resistant to TFT because they cannot incorporate this toxic analog of thymidine into DNA. Cells which grow to form colonies in the presence of TFT are therefore assumed to have mutated, either spontaneously or induced by the test article, at the $TK^{-/-}$ locus.

MATERIALS AND METHODS

Test System

Test Cells. The mouse lymphoma L5178Y cell line, heterozygous at the TK locus and designated as clone 3.7.2C, was used for this assay. Stock cultures were obtained from Dr. Donald Clive and are stored in liquid nitrogen. Laboratory cultures for the mutation assays were maintained in logarithmic growth by serial subculturing for up to 4 months and were then replaced by cells from the frozen stock. Cultures were grown in a shaker incubator at approximately 37°C. A continuous cell log was kept to record growth, doubling times, and subculture operations. To reduce the frequency of spontaneous $TK^{-/-}$ mutants prior to use in the mutation assay, cell cultures were exposed to conditions that selected against the $TK^{-/-}$ phenotype and then returned to normal growth medium for 3 to 8 days.

Media and Cell Culture Conditions. The medium used for this study was RPMI 1640 (Amacher *et al.*, 1980; Clive *et al.*, 1987) supplemented with horse serum (10% by volume), Pluronic® F68, L-glutamine, sodium pyruvate, penicillin and streptomycin. Treatment medium was Fischer's medium with the same medium supplements used in the culture medium except that the horse serum concentration was reduced to 5% by volume. Cloning medium consisted of the RPMI 1640 culture medium with up to 20% horse serum, without Pluronic® F68 and with the addition of 0.24% BBL® agar to achieve a semisolid state. Selection medium was cloning medium that contained 3 µg/mL of TFT (Clive *et al.*, 1987).

Test for Mycoplasma Contamination. Mycoplasma testing was performed by a commercial laboratory on stock cultures prior to preparing stocks for freezing. Stock cultures for the mutation assays were maintained up to 4 months after thawing a sample from a frozen stock. Mycoplasma testing was also performed on cultures thawed for use in assays prior to discarding the stock. This verified that there were no mycoplasma during the life of the culture. Both direct culturing methods and the indirect Hoechst staining method were used.

Karyotype Stability. Karyotype stability, as measured by mean chromosomal number, was routinely performed on stock cultures used for assays. Mean chromosomal number was determined prior to discarding the stock to ensure that changes in the culture had not occurred. Karyotype analysis, including banding, was performed on cells after preparation of a fresh frozen stock culture.

Test Article

The sponsor was responsible for the determination of the test article stability and the test article characteristics as defined in the GLP regulations.

Control Articles

Vehicle Control Article. The vehicle of choice for the test article was DMSO (Sigma Chemical Co., Lot No. 99H0020, CAS #67-68-5). Concurrent vehicle controls were performed for each portion of the assay by exposing the cells to DMSO in culture medium. In the activation portion of the assays, the vehicle controls were also exposed to the S9 metabolic activation mix. Single vehicle control cultures were initiated in the dose rangefinding assays and three vehicle control cultures were initiated in the mutation assays.

Positive Control Articles. The positive control articles listed below were chosen because of the large data base available and because both chemicals induce small and large colonies (Clive *et al.*, 1987, Young *et al.*, 1991).

Methyl methanesulfonate (MMS) is a direct acting mutagen that is highly mutagenic to L5178Y TK⁺ cells. MMS (Aldrich Chemical Co., Lot No. 08109BU, CAS # 66 27-3) was used in duplicate at 13 µg/mL for the nonactivation assay with a 4-hour treatment period, and in duplicate at a concentration of 6.5 µg/mL for the nonactivation assay with a 24-hour treatment period.

Methylcholanthrene (MCA) requires metabolic activation by microsomal enzymes to become mutagenic to L5178Y TK⁺ cells. MCA (Sigma Chemical Co., Lot No. 77H2515, CAS # 56-49-5) was used at 2 µg/mL and 4 µg/mL as a positive control for assays performed with S9 metabolic activation.

S9 Metabolic Activation System

The *in vitro* metabolic activation system was comprised of rat liver enzymes (S9 fraction) and an energy producing system (CORE) comprised of nicotinamide adenine dinucleotide phosphate (NADP, sodium salt) and isocitrate. The S9 enzymes were prepared commercially (Molecular Toxicology, Inc., Boone, NC) and were tested for sterility and enzyme activity. The enzymes were obtained from the 9000 x g supernatant (S9) of liver homogenate from male Sprague Dawley rats treated with Aroclor 1254, which induces mixed function oxidase enzymes capable of transforming chemicals to more active forms. Rats were treated once with 500 mg/kg of Aroclor 1254, and the S9 was prepared approximately 5 days later. The S9 and reaction mixture (CORE) were retained frozen at about -80°C until used.

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S9 Metabolic Activation System

Component	Final Concentration in Cultures
NADP (sodium salt)	3 mM
Isocitrate	15 mM
S9 homogenate	~10 μ L/mL

Dose Ranging Assay

A preliminary dose ranging cytotoxicity experiment was performed with a treatment period of approximately 4 hours with and without S9 activation, since substantial shifts in toxicity often occur for the two test conditions. In addition, a nonactivation assay with a treatment period of approximately 24 hours was performed. A wide range of test article concentrations was tested for cytotoxicity starting at 1250 μ g/mL, and followed by nine lower concentrations prepared in 2-fold dilution steps. After an exposure time of approximately 4 hours or approximately 24 hours at 35-38°C in an orbital shaker at 80 ± 10 orbits per minute, the cells were washed twice with culture medium and resuspended in culture medium. The cells receiving the 24-hour treatment were counted and subcultured, as necessary. Following the termination of the treatment periods, the cells were incubated overnight using procedures identical to those for mutation experiments. A cell count was determined after the growth period to measure the reduction in cell growth relative to the concurrent vehicle control cell cultures. The preliminary dose ranging information was then used to select dose levels for the mutation assay.

Test article concentrations are chosen to cover a toxicity range from 10% to 20% survival to no apparent effect on growth compared to the vehicle control. If little or no toxicity is observed and solubility is maintained, the mutation experiment is initiated with a maximum concentration of 5 mg/mL or 10 mM (whichever is lowest). If precipitation of the test article occurs in the culture medium, the maximum applied dose is at least twice the solubility limit in culture medium. In this assay, the high dose is limited by cytotoxicity.

Mouse Lymphoma Mutagenicity Assay

The assay procedure used was based on that reported by Clive and Spector (1975), Clive *et al.* (1979), Amacher *et al.* (1980) and Clive *et al.* (1987). Although ten or eleven doses were selected to initiate a mutation experiment, the objective was to carry at least four doses through the entire assay. This procedure compensated for normal variations in cellular toxicity and helped to ensure the choice of at least four doses over a wide cytotoxicity range.

Initial Nonactivation Assay. Logarithmically growing laboratory stock cultures were seeded into a series of tubes at 6×10^6 cells per tube. The cells were pelleted by centrifugation, the culture medium removed, and the cells resuspended to a final volume of 10 mL of treatment medium. The tubes were placed in an orbital shaker incubator at 35-38°C and rotated at 80 ± 10 orbits per minute. After an exposure period of approximately 4 hours, the cells were

centrifuged and the treatment medium removed. The cells were then washed, resuspended in 20 mL of culture medium and returned to the orbital shaker incubator.

The assay conditions consisted of vehicle controls in triplicate, two positive controls and eleven different test article dose levels using one culture per dose level. Treated cultures may be eliminated during the expression period as long as four dose levels are left for analysis of mutant induction.

The appearance of the treated cultures was recorded both at the time of treatment and after the treatment period. When the pH indicator in the medium had a color change, the pH was measured using pH indicator strips.

A standard expression period of 2 days was used to allow for mutant recovery, growth and expression of the TK^{-/-} phenotype. Cell densities were determined on Day 1 and were adjusted to 3×10^5 cells/mL in 20 mL of growth medium. If the cells in a culture failed to multiply to a density of 4×10^5 cells/mL on the first day after treatment, the culture was not subcultured. On Day 2, cell counts were again determined, and appropriate cultures were selected for cloning and mutant selection.

Six doses were selected for mutant analysis. If possible, doses were selected to include nontoxic to highly toxic treatment conditions. Although relative growth gives an indication of whether a test article is highly or weakly cytotoxic, specific ranges for weak, moderate, highly toxic and excessively toxic are not specified. Several factors go into the calculation and the Study Director makes a decision concerning the level of cytotoxicity that has occurred. Cultures with cell densities less than approximately 3×10^5 cells/mL were not considered for selection.

A total of 3×10^6 cells from each selected tube was suspended in selection medium in soft agar to recover TFT-resistant mutants. This sample was distributed into three 100 mm dishes. The absolute selection cloning efficiency was determined by seeding three dishes with a total of approximately 600 cells in agar cloning medium. All dishes were placed in an incubator at 35-38°C with 4-6% CO₂:95% humidified air. After 13 days in the incubator, the colonies were counted with the Loats Associates, Inc. (LAI) High Resolution Colony Counter (HRCC) System for the Mouse Lymphoma Assay, Version 1.05b04c.

The mutant frequency was calculated as the ratio of the total number of mutant colonies found in each set of three mutant selection dishes to the total number of cells seeded, adjusted by the absolute selection cloning efficiency. If one dish in either set was lost due to contamination or other cause, the colony count of the missing dish was determined by a proportion equation based upon the weights of the three dishes of the set and the colony counts in the two acceptable dishes. If a lost plate was not available for weighing, the colony count of the lost plate was determined from the average of the two remaining acceptable plates.

The measurement of the cytotoxicity of each treatment was the relative suspension growth of the cells over the 2-day expression period (for the 4-hour treatment) or the relative suspension growth over the 3-day treatment and expression period (for the 24-hour treatment) multiplied by

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the relative cloning efficiency at the time of selection. Although not strictly a measure of cell survival, this parameter (called relative growth or relative total growth, RTG) provides a measure of the effectiveness of treatment and was used as the basis for selecting doses for any necessary subsequent trials.

Confirmatory Nonactivation Assay. The confirmatory assay procedure was similar to the assay using a treatment period of approximately 4 hours except that the exposure time was increased to approximately 24 hours. The assay was conducted using results from the dose rangefinding assay with a treatment period of approximately 24 hours. Ten treatments were initiated, and seven were analyzed for mutant induction.

The cells were obtained from logarithmically growing laboratory stock cultures and were seeded into a series of tubes at 6×10^6 cells per tube. The cells were pelleted by centrifugation, the culture medium removed, and the cells resuspended in a final volume of 20 mL of treatment medium. The dosed tubes were closed, vortexed and placed in an orbital shaker incubator at 35-38°C at 80 ± 10 orbits per minute. After a treatment period of approximately 24 hours, the cells were centrifuged and the treatment medium removed. The cells were then washed, and resuspended in 20 mL of growth medium at 3×10^5 cells per mL. The cultures were then returned to the orbital shaker.

A standard expression period of 2 days following termination of treatment was used to allow recovery, growth and expression of the TK^{-/-} phenotype. Cell densities were determined the day after treatment termination, and each culture was adjusted to 3×10^5 cells/mL in 20 mL of growth medium. If the cells in a culture failed to multiply to a density of 4×10^5 /mL on the first day after treatment termination, the culture was returned to the incubator without being subcultured. The next day cell counts again were determined, and appropriate cultures were selected for cloning and mutant selection. Cultures were counted 12 days after cloning.

Initial Activation Assay. The activation assay was identical to the nonactivation assay except for the addition of the S9 fraction of rat liver homogenate and necessary cofactors (CORE) during the treatment period of approximately 4 hours. The treatment medium included this S9 activation mix, which was prepared immediately prior to use and kept chilled. Each batch of S9 was tested for activation of the MCA positive control article before it was used for the mutagenicity assay. The S9 homogenate was commercially prepared (Molecular Toxicology, Inc., Boone, N.C., Lot No. 955) and consisted of the 9000 x g supernatant from the homogenized livers of Aroclor 1254-induced adult male Sprague Dawley rats. Cultures were counted 13 days after cloning. Eleven treatments were initiated, and four were cloned for mutant analysis.

Confirmatory Activation Assay. The procedures used in the confirmatory activation assay were identical to the initial activation assay. Eleven treatments were initiated, and eight were cloned for mutant analysis. Cultures were counted 13 days after cloning.

Sizing Analysis. The L5178Y TK^{+/-} mutation assay produces a bimodal distribution of large and small mutant colonies. The origin of the bimodal distribution of mutant colony sizes is

considered to reflect the types of genetic damage, with the large colonies derived from cells with intragenic mutations that affect only the TK gene and the small colonies the result of larger mutations that affect cell growth as well as the TK gene (Hozier *et al.*, 1985).

Both the small and large colonies were quantified for all cultures. A bimodal curve is generated and small and large colonies are quantitated by the areas under the curves. The large colonies presumably arise from point mutations and the small colonies from chromosome changes. The LAI Automated Colony Counter was used. This counter sizes all analyzed cultures.

DATA

Data Presentation

The historical control data are presented after the data tables. This report includes the following information:

Dose Rangefinding Data. These data included, for each treatment condition, the daily cell densities of the individual cultures and the cell densities relative to the vehicle controls.

Cell Growth Characteristics. This included the number of cells seeded for the analysis of mutant frequencies, the phenotypic expression time, and the selective agent and concentration. In addition, daily cell densities in the individual cultures carried through the expression period and cloning were included. The suspension growth for vehicle and positive controls, for the 4-hour treatment studies calculated as $(\text{Day 1 cell density}/3 \times 10^5) \times (\text{Day 2 cell density}/3 \times 10^5 \text{ or Day 1 density if not split back})$ describes the growth of the control cultures during the expression period. For the nonactivation assay with a 24-hour treatment period, the suspension growth for vehicle and positive controls, calculated as $[\text{Treatment termination (Day 1) cell density}/3 \times 10^5] \times [\text{Day 2 cell density}/3 \times 10^5 \text{ or Day 1 density if not split back}] \times [\text{Day 3 cell density}/3 \times 10^5 \text{ or Day 2 density if not split back}]$, describes the growth of the control cultures during the expression period. For the test-article treated cultures, the relative suspension growths were included and calculated as above, and were expressed as percentages of the average vehicle control suspension growth. The relative cloning efficiencies of test article-treated cultures were expressed as percentages of the average vehicle control cloning efficiency. The expression of RTG was the parameter that was used to assess treatment cytotoxicity and was obtained by multiplying the relative suspension growth by the relative cloning efficiency/100.

Mutant Selection Data. The mutant selection data includes the total mutant colonies (i.e. total number of mutant colony counts obtained from 3×10^6 cells sampled from one culture, seeded into selective medium and divided among three culture dishes) and the total viable colonies (i.e. the total number of colony counts obtained from 600 cells sampled from one culture, seeded into nonselective medium and divided among three culture dishes). These were used to calculate the mutant frequency for each culture. The ratio of cells seeded for mutant selection to cells seeded for cloning efficiency was 0.5×10^4 . Therefore, the mutant frequency was: (total mutant

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250
255

colonies/total viable colonies) $\times 2 \times 10^{-4}$. Mutant frequency was given in units of 10^{-6} . Also included in this section was the cloning efficiency of vehicle and positive controls calculated as (total number of viable colonies/600) $\times 100\%$.

Assay Acceptance Criteria

An assay was considered acceptable for evaluation of the test results only if all of the criteria given below were satisfied. Activation or nonactivation assays were repeated independently, as needed, to satisfy acceptance criteria. The Study Director reviewed each assay in accordance with the following criteria:

Acceptable Controls. The average absolute cloning efficiency of the vehicle controls should be between 60% and 130%. A value greater than 100% is possible because of errors in cell counts (usually $\pm 10\%$) and variations in cell division during unavoidable delays between counting and cloning of many cultures.

A minimum acceptable value for the average suspension growth of the vehicle controls for 2 days is an 8.0-fold increase over the original cell concentrations. Lower values render an assay unacceptable because of poor cell growth.

The background mutant frequency (average of vehicle control values) is calculated separately for concurrent activation and nonactivation assays, even though the same population of cells is used for each condition. For both conditions, the normal range of background frequencies for assays performed with different cell stocks is 30×10^{-6} to 120×10^{-6} . Assays with backgrounds outside this range are not necessarily invalid but will be evaluated with caution.

At least one of the positive control cultures in each trial should induce a mutant frequency of at least 200×10^{-6} . A trial may be acceptable without a positive control (due to contamination or technical error) only if the test article is positive.

Acceptable High Dose. For test articles with weak or no mutagenic activity, an assay should include applied concentrations that reduce the RTG to 10% or 20% of the average vehicle control or reach the maximum applied concentrations given in the evaluation criteria. RTG represents a calculation of survival that is based on both relative suspension growth during the expression period and relative cloning efficiency at the time of plating. Because mutant frequencies increase as a function of lethality, an attempt to obtain treatments in the range of 10% to 20% RTG must be made in order to consider the assay as conclusive. This requirement was waived if the concentration of the highest assayed dose was at least 75% of a higher, excessively cytotoxic dose level or if the highest dose was at least twice the solubility limit of the test article in culture medium. There is no maximum toxicity requirement for test articles which clearly show mutagenic activity.

Acceptable Number of Doses. Mutant frequency will be considered acceptable for evaluation only if the relative cloning efficiency is 10% or greater and the total number of viable clones exceeds about 60. These limits avoid problems with the statistical distribution of scorable colonies among dishes.

Mutant frequencies for at least four different test article dose levels are normally determined in each assay; a minimum of four analyzed cultures is considered necessary to accept a single assay for evaluation of the test article.

Mutant frequencies are normally derived from sets of three dishes for both mutant colony count and viable colony count. In order to allow for losses due to contamination or other reasons, an acceptable mutant frequency can be calculated from a minimum of two dishes per set.

Assay Evaluation Criteria

The test article was evaluated as positive, negative, or equivocal in this assay. A positive evaluation indicates that the test article is a mutagen (induces gene/chromosomal mutations) in this test system. A negative evaluation indicates the test article is nonmutagenic in this test system and causes no responses that can be interpreted as positive. However, there are substances for which the results remain questionable; any test article in this category will be evaluated as equivocal in this test system.

Evaluation of a Positive Response. The test article is evaluated as positive if dose-dependent increases of 2-fold or greater in mutant frequency are obtained over the concurrent background mutant frequency. The background mutant frequency is defined as the average mutant frequency of the vehicle control cultures. The 2-fold or greater increase is based on extensive experience which indicates such responses are repeatable in additional trials. It is desirable to obtain this relationship for at least three doses, but this goal depends on the dose steps chosen for the assay and toxicity at which mutagenic activity appears.

The dose-dependent requirement is waived if a large increase in mutant frequency (4-fold or higher) is obtained for a single dose at or near the highest testable toxicity. However, for the test article to be evaluated as positive, any increases must be repeatable in a second trial.

Evaluation of a Negative Response. The test article was evaluated as negative if a 2-fold increase in mutant frequency was not observed for (1) a range of doses that extended to toxicities causing 10% to 20% RTG, or (2) for relatively nontoxic test articles, a range of doses extending to the maximum concentration of 5 mg/mL or 10 mM (whichever is lower), or (3) a range of doses that extended to a level approximately twice the solubility limit in culture medium, or (4) the increase(s) are not repeatable in a confirmatory trial.

Evaluation of an Equivocal Response. The test article was evaluated as equivocal in this test system if there was no consistent evidence for either a positive or negative evaluation.

Other Considerations. Treatments with test articles that reduced the RTG to less than 10% may occur in an assay, but the results obtained at this high toxicity were considered biologically irrelevant and were not used to evaluate the test article.

For some test articles, the correlation between toxicity and applied concentration is poor. The interaction of the test article with the cells is not always repeatable or controlled by

concentration. In this case, the induced toxicity as measured by the RTG is a better measure of a dose related response than applied concentration. Therefore, in determining if a dose-relationship is present, either test article concentration or induced toxicity (RTG) may be used.

This presentation of the evaluation process may not encompass all test situations, and the Study Director may have used other criteria to arrive at an evaluation. The report provides the reasoning involved when departures from the above description occur.

RESULTS

Test Article Handling

The test article formed unworkable, non-homogeneous suspensions in water at concentrations of 498, 300, 100 and 50.0 mg/mL. In DMSO, the test article formed a solution at 500 mg/mL. For this reason, DMSO (CAS # 67-68-5, Sigma Chemical Co., Lot No. 99H0020) was used as the vehicle.

N-EtFOSE T-6316 was soluble in the vehicle (DMSO) at 125 mg/mL, the highest concentration prepared for use in the assay. The stock used for dosing the highest concentration of the test article was prepared in DMSO at 100-times the highest desired treatment concentration. Further stocks were prepared by performing serial dilutions in the vehicle. Dosing was initiated by performing 1:100 dilutions of the stocks into medium containing the cells. All dosing was performed with freshly prepared test article stocks.

Dose Rangefinding Assay

The test article, N-EtFOSE T-6316, was tested in a preliminary dose rangefinding assay with a treatment period of approximately 4 hours both with and without S9 metabolic activation and a preliminary dose rangefinding nonactivation assay with a treatment period of approximately 24 hours. Ten dose levels were used in each case that ranged from 2.47 to 1250 µg/mL; a vehicle control was included under each condition.

In the presence and absence of rat liver S9 metabolic activity with a 4-hour treatment period (Table 1), N-EtFOSE T-6316 was noncytotoxic up to 19.7 µg/mL. Moderate cytotoxicity was induced from 39.3 to 157 µg/mL and higher concentrations were excessively cytotoxic or lethal. In the nonactivation dose rangefinding assay using a 24-hour treatment period (Table 2), the test article induced no cytotoxicity at 2.47 µg/mL, weak cytotoxicity at 4.93 µg/mL, and moderately high cytotoxicity at 9.85 µg/mL. Higher concentrations were excessively cytotoxic or lethal. Dose levels for the mutation assays were based on these results.

Mutation Assays

Initial Nonactivation Mutation Assay. Eleven concentrations of N-EtFOSE T-6316 at 25.3, 50.5, 75.5, 101, 151, 201, 251, 301, 401, 501 and 601 µg/mL were initiated. Treatments at and above 251 µg/mL were terminated because of excessive cytotoxicity. The remaining six treatments induced weak cytotoxicity to high cytotoxicity (67.6% to 14.4% relative growths). In

order for a treatment to be evaluated as mutagenic in the initial nonactivation assay, a mutant frequency greater than 127.1×10^{-6} was required. This threshold value was equal to twice the average mutant frequency of the concurrent vehicle controls (background mutant frequency). None of the analyzed treatment induced this level of mutant action (Table 3). Sizing data is shown in Table 4. A confirmatory assay was initiated.

Confirmatory Nonactivation Mutation Assay. In the confirmatory nonactivation assay, the treatment period was approximately 24 hours and results are shown in Table 5. Ten treatments at 1.25, 2.50, 5.00, 7.50, 10.0, 12.5, 15.0, 20.0, 30.0 and 40.0 $\mu\text{g/mL}$ were initiated. Treatments at 20.0, 30.0 and 40.0 $\mu\text{g/mL}$ were terminated because of excessive cytotoxicity. The remaining seven treatments were selected for mutant analysis and induced no cytotoxicity to high cytotoxicity (94.2% to 18.7% relative growths). None of the analyzed treatments induced a mutant frequency that exceeded the minimum criterion of 111.2×10^{-6} . The test article is, therefore, considered negative without metabolic activation. Sizing data for the confirmatory assay is shown in Table 6.

Initial Activation Mutation Assay. Eleven treatments at 25.3, 50.5, 75.5, 101, 151, 201, 251, 301, 401, 501 and 601 $\mu\text{g/mL}$ were initiated, and treatments at and above 151 $\mu\text{g/mL}$ were terminated because of excessive cytotoxicity. The remaining four doses were cloned for mutant analysis and induced moderate to very high cytotoxicity (60.0% to 9.4% relative growths). The minimum criterion for a positive response in this trial was 156.7×10^{-6} . None of the analyzed treatments induced this level of mutant action (Table 7). A confirmatory assay was performed. Sizing data for the initial assay is shown in Table 8.

Confirmatory Activation Mutation Assay. In the confirmatory assay with metabolic activation (Table 9), eleven treatments at 5.00, 10.0, 20.0, 30.0, 40.0, 50.0, 60.0, 80.0, 100, 150 and 200 $\mu\text{g/mL}$ were initiated. Treatments at 5.00 and 10.0 $\mu\text{g/mL}$ were terminated because there were sufficient higher doses available for analysis and the 200 $\mu\text{g/mL}$ treatment was terminated because of excessive cytotoxicity. The remaining eight doses induced weak cytotoxicity to high cytotoxicity (70.4% to 15.4% relative growths). None of the analyzed treatments induced a mutant frequency that exceeded the minimum criteria of 122.2×10^{-6} . The test article was evaluated as negative with metabolic activation in this assay. Sizing data for the confirmatory assay is shown in Table 10.

Control Values. The average cloning efficiencies for the vehicle controls were 88.6% and 83.4% without activation and 84.1% and 77.3% with S9 metabolic activation, which demonstrated acceptable cloning conditions for the assays. The positive control cultures, MMS (nonactivation) and MCA (activation) induced large increases in mutant frequency that were greatly in excess of the minimum criteria.

Sizing Analysis. The L5178Y TK^{+/+} mutation assay produces a bimodal distribution of large and small mutant colonies. The origin of the bimodal distribution of mutant colony sizes is considered to reflect the types of genetic damage, with the large colonies derived from cells with intragenic mutations that affect only the TK gene and the small colonies the result of larger mutations that affect cell growth as well as the TK gene. Colony sizing was performed on all

cultures (Tables 4, 6, 8 and 10). Mutant colonies from all the cultures showed the expected bimodal distribution and mutant colonies from MMS and MCA treated cultures showed both small and large colonies.

CONCLUSION

The test article, N-EtFOSE T-6316, was evaluated as negative for inducing forward mutations at the TK locus in L5178Y mouse lymphoma cells under activation and nonactivation conditions used in this study. Treatments up to the testing limit for this assay were analyzed.

RECORDS TO BE MAINTAINED

All raw data, documentation, records, protocol and a copy of the final report generated as a result of this study will be archived in the storage facilities of Covance-Vienna, for at least 1 year following submission of the final report to the Sponsor. After the 1-year period, the Sponsor may elect to have the aforementioned materials retained in the storage facilities of Covance-Vienna for an additional period of time, or sent to a storage facility designated by the Sponsor.

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DATA TABLES

TABLE 1: CYTOTOXICITY ASSAY WITH N-EtFOSE T-6316 (4-HOUR)

TEST ARTICLE: N-EtFOSE T-6316

STUDY NUMBER: 20785-0-431 ICH

TEST DATE: 05/10/2000

VEHICLE: DMSO

COMMENTS ON TREATMENT: ~4-Hour Treatment Period

APPLIED CONCENTRATION $\mu\text{g/mL}$	WITHOUT S9 ACTIVATION		WITH S9 ACTIVATION	
	CELL DENSITY/mL ($\times 10^5$) ^a	% VEHICLE CONTROL ^b	CELL DENSITY/mL ($\times 10^5$) ^a	% VEHICLE CONTROL ^b
VC ^c	10.0	100.0	10.4	100.0
2.47	9.2	92.0	10.8	103.8
4.93	10.8	108.0	9.4	90.4
9.85	11.8	118.0	11.7	112.5
19.7	9.1	91.0	9.2	88.5
39.3	4.7	47.0	5.1	49.0
78.5	3.3	33.0	2.8	26.9
157	3.5	35.0	2.4	23.1
313	0.4	4.0	0.3	2.9
625	0.0	0.0	0.0	0.0
1250	0.0	0.0	0.0	0.0

^aCell density determined by hemocytometer

^bRelative to vehicle control cell density for all treatments

^cVC = Vehicle control, 1% DMSO

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TABLE 2: CYTOTOXICITY ASSAY WITH N-EtFOSE T-6316
WITHOUT S9 ACTIVATION (24-HOUR)

TEST ARTICLE: N-EtFOSE T-6316

STUDY NUMBER: 20785-0-431 ICH

TEST DATE: 05/10/00

VEHICLE: DMSO

COMMENTS ON TREATMENT: ~24-Hour Treatment Period

APPLIED CONCENTRATION mg/mL	CELL DENSITY/ML (X10 ⁵) ^a		% VEHICLE CONTROL ^b
	DAY 1	DAY 2	
VC ^c	11.0	7.0	100.0
2.47	12.1	6.4	100.6
4.93	7.2	7.3	68.3
9.85	5.7	3.3	24.4
19.7	0.3*	1.0	3.9
39.3	0.0*	0.0	0.0
78.5	0.0*	0.0	0.0
157	NTC	NTC	---
313	NTC	NTC	---
625	NTC	NTC	---
1250	NTC	NTC	---

^aCell density determined by hemocytometer

^bRelative to vehicle control cell density for all treatments

^cVC = Vehicle control, 1% DMSO

NTC = Not counted, cytotoxic

*Not subcultured

TABLE 3: INITIAL MUTATION ASSAY WITHOUT ACTIVATION

A. TEST ARTICLE: N-EtFOSE T-6316
B. GENETICS ASSAY NO.: 20785-0-431 ICH
C. VEHICLE: DMSO
D. SELECTIVE AGENT: TFT 3.0 µg/mL

E. TREATMENT DATE: 05/16/2000
F. CELLS ANALYZED: 3x10⁶
G. TREATMENT PERIOD: ~4 hours
H. EXPRESSION PERIOD: 2 days

Test Condition	Daily Cell Counts (Cell/mL, 10E5 Units)		Cumulative RSG ^a	Total Mutant Colonies	Total Viable Colonies	Cloning Efficiency ^b	Relative Growth (%) ^c	Mutant Frequency (10E-6 Units) ^d
	Day 1	Day 2						
Nonactivation Controls ^e			AVG VC			AVG VC		
Vehicle Control	10.0	14.2	15.8	188	501	83.5	94.5	74.9
Vehicle Control	12.2	11.5	15.6	157 ^h	544	90.7	101.5	57.5
Vehicle Control	11.7	12.2	15.9	160 ^h	549	91.5	88.6	58.2
MMS 13 µg/mL	6.9	12.4	9.5	389 ^h	271	45.1	30.8	287.9 ^f
MMS 13 µg/mL	8.2	12.0	10.9	395	225 ^h	37.5	29.4	350.8 ^f
Test Compound µg/mL			Relative to Vehicle Control (%)			Relative to Vehicle Control (%)		
25.3	7.8	14.2	78.2	183	459	86.4	67.6	79.8
50.5	4.6	10.6	34.4	170 ^h	483	91.0	31.3	70.2
75.5	2.8 ^g	9.7	20.5	142	469	88.3	18.1	60.5
101	1.3 ^g	8.2	17.4	171	465	87.5	15.2	73.7
151	2.1 ^g	8.5	18.0	145	485	91.4	16.4	59.8
201	2.0 ^g	8.0	16.9	173	451	84.8	14.4	77.0

^aRSG = (Day 1 Count/3) * (Day 2 Count)/3 (or Day 1 Count if not subcultured)

^bCloning Efficiency = Total Viable Colony Count/Number of Cells Seeded * 100

^cRelative Growth = (Relative Suspension Growth * Relative Cloning Efficiency) / 100

^dMutant Frequency = (Total Mutant Colonies/Total Viable Colonies) * 2x10E-4

Decimal is moved to express the frequency in units of 10E-6

^eVehicle Control = 1% DMSO

Positive Control: MMS = Methyl methanesulfonate

^fMutagenic. Exceeds Minimum Criterion of 127.1 X 10E-6

^gNot Subcultured

^hOne plate contaminated. Total counts calculated by using a weight proportion

TABLE 4: SIZING DATA FOR INITIAL MUTATION ASSAY WITHOUT ACTIVATION

A. TEST ARTICLE: N-EtFOSE T-6316
B. GENETICS ASSAY NO.: 20785-0-431 ICH
C. VEHICLE: DMSO
D. SELECTIVE AGENT: TFT 3.0 µg/mL
E. TREATMENT DATE: 05/16/2000

Test Condition	Conc.	Cum. RSG (%) ^a		Cloning Efficiency ^b		Relative Growth ^c (%)	Mutant Frequency (x10 ⁻⁶) ^d		
		Day 1	Day 2	Abs %	Rel %		Total	Small	Large
Vehicle Control ^e									
	1%	88.5	100.2	83.5	94.3	94.5	74.9	42.3	32.7
	1%	108.0	99.0	90.7	102.5	101.5	57.5	43.0	14.5
	1%	103.5	100.7	91.5	103.3	104.1	58.2	34.8	23.4
MMS ^f (µg/mL)									
	13	61.1	60.4	45.1	50.9	30.8	287.9	214.1	73.8
	13	72.6	69.5	37.5	42.4	29.4	350.8	169.6	181.2
Test Article(µg/mL)									
	25.3	69.0	78.2	76.6	86.4	67.6	79.8	40.9	39.0
	50.5	40.7	34.4	80.6	91.0	31.3	70.2	42.8	27.4
	75.5	24.8	20.5	78.2	88.3	18.1	60.5	34.0	26.5
	101	11.5	17.4	77.5	87.5	15.2	73.7	44.6	29.1
	151	18.6	18.0	80.9	91.4	16.4	59.8	35.1	24.7
	201	17.7	16.9	75.1	84.8	14.4	77.0	47.9	29.1

^aCum. RSG = Cumulative Suspension Growth Relative to the Average Vehicle Control Suspension Growth

^bCloning Efficiency = Total Viable Colony Count/Number of Cells Seeded * 100

^cRelative Growth = (Relative Suspension Growth * Relative Cloning Efficiency) / 100

^dMutant Frequency = (Total Mutant Colonies/Total Viable Colonies) * 2x10E-4

Decimal is moved to express the frequency in units of 10E-6

Expressed as Total Mutant Frequency, Small Colony Mutant Frequency and Large Colony Mutant Frequency

^eVehicle Control = DMSO

^fPositive Control: MMS = Methyl methanesulfonate

Colony Counts increased by 9.099% to compensate for area of dish not scanned

TABLE 5: CONFIRMATORY MUTATION ASSAY WITHOUT ACTIVATION

A. TEST ARTICLE: N-EtFOSE T-6316
B. GENETICS ASSAY NO.: 20785-0-431 ICH
C. VEHICLE: DMSO
D. SELECTIVE AGENT: TFT 3.0 µg/mL

E. TREATMENT DATE: 06/07/2000
F. CELLS ANALYZED: 3×10^6
G. TREATMENT PERIOD: ~24 hours
H. EXPRESSION PERIOD: 2 days

Test Condition	Daily Cell Counts (Cell/mL, 10E5 Units)			Cumulative RSG ^a	Total Mutant Colonies	Total Viable Colonies	Cloning Efficiency ^b	Relative Growth (%) ^c	Mutant Frequency (10E-6 Units) ^d
	Day 1	Day 2	Day 3						
Nonactivation Controls ^e				AVG VC			AVG VC		
Vehicle Control	12.0	7.9	12.0	43.1	133	464	77.3	87.4	57.4
Vehicle Control	14.3	6.2	11.4	37.4	154	597	99.5	99.9	51.6
Vehicle Control	14.7	6.1	16.4	54.5	44.7	128	441	73.5	83.4
MMS 6.5 µg/mL	9.5	4.9	6.8	11.7	571	211	35.1	11.0	542.0 ^f
MMS 6.5 µg/mL	9.6	4.5	5.2	8.3	574	233	38.9	8.7	491.6 ^f
Test Compound µg/mL				Relative to Vehicle Control (%)			Relative to Vehicle Control (%)		
1.25	10.0	7.5	10.6	65.9	173	568	113.6	74.9	61.0
2.50	9.3	6.6	16.2	82.4	136	463	92.4	76.2	59.0
5.00	12.1	9.1	12.2	111.4	158	423	84.6	94.2	74.7
7.50	9.4	6.5	15.7	79.5	147	433	86.6	68.8	68.0
10.0	9.6	7.0	10.8	60.2	148	552	110.3	66.4	53.8
12.5	6.6	5.9	12.2	39.4	133	424	84.8	33.4	62.7
15.0	6.3	3.9 ^g	13.5	21.2	169	442	88.3	18.7	76.5

^aRSG = [Treatment termination (Day 1) cell density/ 3×10^5] x [Day 2 cell density/ 3×10^5 or Day 1 density if not split back] x [Day 3 cell density/ 3×10^5 or Day 2 density if not split back]

^bCloning Efficiency = Total Viable Colony Count/Number of Cells Seeded * 100

^cRelative Growth = (Relative Suspension Growth * Relative Cloning Efficiency) / 100

^dMutant Frequency = (Total Mutant Colonies/Total Viable Colonies) * 2×10^{-4}

Decimal is moved to express the frequency in units of 10E-6

^eVehicle Control = 1% DMSO

Positive Control: MMS = Methyl methanesulfonate

^fMutagenic. Exceeds Minimum Criterion of 111.2×10^{-6}

^gNot subcultured

TABLE 6: SIZING DATA FOR CONFIRMATORY MUTATION ASSAY WITHOUT ACTIVATION

A. TEST ARTICLE: N-EtFOSE T-6316
B. GENETICS ASSAY NO.: 20785-0-431 ICH
C. VEHICLE: DMSO
D. SELECTIVE AGENT: TFT 3.0 µg/mL
E. TREATMENT DATE: 06/07/2000

Test Condition	Conc.	Cum. RSG (%) ^a			Cloning Efficiency ^b		Relative Growth ^c (%)	Mutant Frequency (x10 ⁻⁶) ^d		
		Day 1	Day 2	Day 3	Abs %	Rel %		Total	Small	Large
Vehicle Control ^e										
	1%	87.8	104.1	94.3	77.3	92.7	87.4	57.4	24.5	32.9
	1%	104.6	97.4	83.8	99.5	119.3	99.9	51.6	24.5	27.1
	1%	107.6	98.5	121.9	73.5	88.1	107.4	57.9	32.2	25.7
MMS ^f (µg/mL)										
	6.5	69.5	51.1	26.2	35.1	42.1	11.0	542.0	336.8	205.2
	6.5	70.2	47.4	18.6	38.9	46.7	8.7	491.6	316.8	174.8
Test Article(µg/mL)										
	1.25	73.2	82.4	65.9	94.7	113.6	74.9	61.0	29.9	31.1
	2.50	68.0	67.4	82.4	77.1	92.4	76.2	59.0	27.8	31.1
	5.00	88.5	120.9	111.4	70.6	84.6	94.2	74.7	37.1	37.6
	7.50	68.8	67.1	79.5	72.2	86.6	68.8	68.0	34.3	33.8
	10.0	70.2	73.8	60.2	92.0	110.3	66.4	53.8	31.2	22.5
	12.5	48.3	42.8	39.4	70.7	84.8	33.4	62.7	35.0	27.8
	15.0	46.1	20.8	21.2	73.6	88.3	18.7	76.5	39.5	37.0

^aCum. RSG = Cumulative Suspension Growth Relative to the Average Vehicle Control Suspension Growth

^bCloning Efficiency = Total Viable Colony Count/Number of Cells Seeded * 100

^cRelative Growth = (Relative Suspension Growth * Relative Cloning Efficiency) / 100

^dMutant Frequency = (Total Mutant Colonies/Total Viable Colonies) * 2x10E-4

Decimal is moved to express the frequency in units of 10E-6

Expressed as Total Mutant Frequency, Small Colony Mutant Frequency and Large Colony Mutant Frequency

^eVehicle Control = DMSO

^fPositive Control: MMS = Methyl methanesulfonate

Colony Counts increased by 9.099% to compensate for area of dish not scanned

TABLE 7: INITIAL MUTATION ASSAY WITH ACTIVATION

A. TEST ARTICLE: N-EtFOSE T-6316

B. GENETICS ASSAY NO.: 20785-0-431 ICH

C. VEHICLE: DMSO

D. SELECTIVE AGENT: TFT 3.0 µg/mL

E. TREATMENT DATE: 05/16/2000

F. CELLS ANALYZED: 3×10^6

G. TREATMENT PERIOD: ~4 hours

H. EXPRESSION PERIOD: 2 days

Test Condition	Daily Cell Counts (Cell/mL, 10E5 Units)		Cumulative RSG ^a	Total Mutant Colonies	Total Viable Colonies	Cloning Efficiency ^b	Relative Growth (%) ^c	Mutant Frequency (10E-6 Units) ^d
	Day 1	Day 2						
S9-Activation Controls ^e S9 Batch Number: 955			AVG VC			AVG VC		
Vehicle Control	12.7	17.3	24.4	170 ^h	437	72.9	112.5	77.9
Vehicle Control	11.1	15.0	18.5	201	503	83.8	98.0	79.8
Vehicle Control	10.8	11.3	13.6	221	573	95.5	84.1	77.3
MCA 2 µg/mL	7.6	15.1	12.8	605	381	63.5	51.1	318.1 ^f
MCA 4 µg/mL	6.2	15.6	10.7	607	391	65.1	44.2	310.6 ^f
Test Compound µg/mL			Relative to Vehicle Control			Relative to Vehicle Control		
25.3	8.8	11.9	61.8	167	490	97.1	60.0	68.2
50.5	3.1 ^g	13.0	23.0	149	476	94.3	21.7	62.8
75.5	1.6 ^g	7.5	13.3	178	490	97.1	12.9	72.6
101	0.5 ^g	6.2	11.0	167	433	85.9	9.4	77.1

^aRSG = (Day 1 Count/3) * (Day 2 Count)/3 (or Day 1 Count if not subcultured)

^bCloning Efficiency = Total Viable Colony Count/Number of Cells Seeded * 100

^cRelative Growth = (Relative Suspension Growth * Relative Cloning Efficiency) / 100

^dMutant Frequency = (Total Mutant Colonies/Total Viable Colonies) * 2×10^{-4}

Decimal is moved to express the frequency in units of 10E-6

^eVehicle Control = 1% DMSO

Positive Control: MCA = Methylcholanthrene

^fMutagenic. Exceeds Minimum Criterion of 156.7×10^{-6}

^gNot subcultured

^hOne plate contaminated. Total counts calculated by using a weight proportion

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TABLE 8: SIZING DATA FOR INITIAL MUTATION ASSAY WITH ACTIVATION

A. TEST ARTICLE: N-EtFOSE T-6316
B. GENETICS ASSAY NO.: 20785-0-431 ICH
C. VEHICLE: DMSO
D. SELECTIVE AGENT: TFT 3.0 µg/mL
E. TREATMENT DATE: 05/16/2000

Test Condition	Conc.	Cum. RSG (%) ^a		Cloning Efficiency ^b		Relative Growth ^c (%)	Mutant Frequency (x10 ⁻⁶) ^d		
		Day 1	Day 2	Abs %	Rel %		Total	Small	Large
Vehicle Control ^e	1%	110.1	129.7	72.9	86.7	112.5	77.9	35.3	42.6
	1%	96.2	98.3	83.8	99.7	98.0	79.8	49.9	29.9
	1%	93.6	72.0	95.5	113.6	81.8	77.3	46.9	30.5
MCA ^f (µg/mL)	2	65.9	67.7	63.5	75.5	51.1	318.1	150.1	167.9
	4	53.8	57.1	65.1	77.4	44.2	310.6	166.5	144.1
Test Article(µg/mL)	25.3	76.3	61.8	81.6	97.1	60.0	68.2	38.8	29.4
	50.5	26.9	23.0	79.3	94.3	21.7	62.8	38.5	24.3
	75.5	13.9	13.3	81.6	97.1	12.9	72.6	42.8	29.8
	101	4.3	11.0	72.2	85.9	9.4	77.1	55.4	21.7

^aCum. RSG = Cumulative Suspension Growth Relative to the Average Vehicle Control Suspension Growth

^bCloning Efficiency = Total Viable Colony Count/Number of Cells Seeded * 100

^cRelative Growth = (Relative Suspension Growth * Relative Cloning Efficiency) / 100

^dMutant Frequency = (Total Mutant Colonies/Total Viable Colonies) * 2x10E-4

Decimal is moved to express the frequency in units of 10E-6

Expressed as Total Mutant Frequency, Small Colony Mutant Frequency and Large Colony Mutant Frequency

^eVehicle Control = DMSO

^fPositive Control: MCA = Methylcholanthrene

Colony Counts increased by 9.099% to compensate for area of dish not scanned

TABLE 9: CONFIRMATORY MUTATION ASSAY WITH ACTIVATION

A. TEST ARTICLE: N-EtFOSE T-6316
B. GENETICS ASSAY NO.: 20785-0-431 ICH
C. VEHICLE: DMSO
D. SELECTIVE AGENT: TFT 3.0 µg/mL

E. TREATMENT DATE: 06/07/2000
F. CELLS ANALYZED: 3×10^6
G. TREATMENT PERIOD: ~4 hours
H. EXPRESSION PERIOD: 2 days

Test Condition	Daily Cell Counts (Cell/mL, 10E5 Units)		Cumulative RSG ^a	Total Mutant Colonies	Total Viable Colonies	Cloning Efficiency ^b	Relative Growth (%) ^c	Mutant Frequency (10E-6 Units) ^d
	Day 1	Day 2						
S9-Activation Controls ^e S9 Batch Number: 955			AVG VC			AVG VC		
Vehicle Control	9.4	16.9	17.7	128	479	79.8	97.9	53.3
Vehicle Control	9.1	15.9	16.1	149	461	76.9	85.9	64.8
Vehicle Control	14.3	13.9	22.1	147	452	75.3	77.3	65.2
MCA 2 µg/mL	7.3	13.7	11.1	472	443	73.8	57.0	213.3 ^f
MCA 4 µg/mL	9.1	12.6	12.7	412	405	67.5	59.7	203.8 ^f
Test Compound µg/mL			Relative to Vehicle Control (%)			Relative to Vehicle Control (%)		
20.0	8.0	19.4	92.7	131	352	75.9	70.4	74.3
30.0	7.5	12.0	53.8	132	525	113.1	60.8	50.3
40.0	5.5	17.0	55.8	128	433	93.3	52.1	58.9
50.0	5.2	14.2	44.1	122	445	95.9	42.3	54.9
60.0	3.4 ^g	14.4	25.8	100	444	95.7	24.7	45.2
80.0	2.1 ^g	13.9	24.9	122	453	97.6	24.3	54.0
100	2.2 ^g	10.5	18.8	135	464	99.9	18.8	58.4
150	2.8 ^g	7.5	13.4	153	532	114.7	15.4	57.4

^aRSG = (Day 1 Count/3) * (Day 2 Count)/3 (or Day 1 Count if not subcultured)

^bCloning Efficiency = Total Viable Colony Count/Number of Cells Seeded * 100

^cRelative Growth = (Relative Suspension Growth * Relative Cloning Efficiency) / 100

^dMutant Frequency = (Total Mutant Colonies/Total Viable Colonies) * 2×10^{-4}

Decimal is moved to express the frequency in units of 10E-6

^eVehicle Control = 1% DMSO

Positive Control: MCA = Methylcholanthrene

^fMutagenic. Exceeds Minimum Criterion of 122.2×10^{-6}

^gNot Subcultured

TABLE 10: SIZING DATA FOR CONFIRMATORY MUTATION ASSAY WITH ACTIVATION

A. TEST ARTICLE: N-EtFOSE T-6316
B. GENETICS ASSAY NO.: 20785-0-431 ICH
C. VEHICLE: DMSO
D. SELECTIVE AGENT: TFT 3.0 µg/mL
E. TREATMENT DATE: 06/07/2000

Test Condition	Conc.	Cum. RSG (%) ^a		Cloning Efficiency ^b		Relative Growth ^c (%)	Mutant Frequency (x10 ⁻⁶) ^d		
		Day 1	Day 2	Abs %	Rel %		Total	Small	Large
Vehicle Control ^e									
	1%	86.0	94.9	79.8	103.2	97.9	53.3	23.7	29.6
	1%	83.2	86.4	76.9	99.5	85.9	64.8	28.4	36.4
	1%	130.8	118.7	75.3	97.3	115.5	65.2	26.6	38.6
MCA ^f (µg/mL)									
	2	66.8	59.7	73.8	95.5	57.0	213.3	86.2	127.1
	4	83.2	68.5	67.5	87.2	59.7	203.8	97.0	106.7
Test Article(µg/mL)									
	20.0	73.2	92.7	58.7	75.9	70.4	74.3	32.8	41.5
	30.0	68.6	53.8	87.5	113.1	60.8	50.3	21.6	28.7
	40.0	50.3	55.8	72.2	93.3	52.1	58.9	29.7	29.2
	50.0	47.6	44.1	74.2	95.9	42.3	54.9	29.4	25.5
	60.0	31.1	25.8	74.0	95.7	24.7	45.2	23.6	21.6
	80.0	19.2	24.9	75.5	97.6	24.3	54.0	28.4	25.5
	100	20.1	18.8	77.3	99.9	18.8	58.4	30.1	28.2
	150	25.6	13.4	88.7	114.7	15.4	57.4	29.5	27.9

^aCum. RSG = Cumulative Suspension Growth Relative to the Average Vehicle Control Suspension Growth

^bCloning Efficiency = Total Viable Colony Count/Number of Cells Seeded * 100

^cRelative Growth = (Relative Suspension Growth * Relative Cloning Efficiency) / 100

^dMutant Frequency = (Total Mutant Colonies/Total Viable Colonies) * 2x10E-4

Decimal is moved to express the frequency in units of 10E-6

Expressed as Total Mutant Frequency, Small Colony Mutant Frequency and Large Colony Mutant Frequency

^eVehicle Control = DMSO

^fPositive Control: MCA = Methylcholanthrene

Colony Counts increased by 9.099% to compensate for area of dish not scanned

HISTORICAL CONTROL DATA

Nonactivation Studies

Pooled negative and vehicle control mutant frequencies

Mean ($\pm$ SD)	$53.0 \pm 22.0 \times 10^{-6}$
Range	20.5 to 114.8×10^{-6}
Number of experiments	52
Number of controls	156

Positive control mutant frequencies (6.5 $\mu\text{g/mL}$ methyl methanesulfonate)

Mean ($\pm$ SD)	$272.7 \pm 135.7 \times 10^{-6}$
Range	115.9 to 632.1×10^{-6}
Number of experiments	51
Number of controls	51

Positive control mutant frequencies (13 $\mu\text{g/mL}$ methyl methanesulfonate)

Mean ($\pm$ SD)	$483.9 \pm 315.2 \times 10^{-6}$
Range	176.1 to 1996.4×10^{-6}
Number of experiments	52
Number of controls	52

Activation Studies

Pooled negative and vehicle control mutant frequencies

Mean ($\pm$ SD)	$65.3 \pm 27.1 \times 10^{-6}$
Range	27.6 to 150.3×10^{-6}
Number of experiments	54
Number of controls	162

Positive control mutant frequencies (2.0 $\mu\text{g/mL}$ 3-methylcholanthrene)

Mean ($\pm$ SD)	$454.5 \pm 166.2 \times 10^{-6}$
Range	204.8 to 787.8×10^{-6}
Number of experiments	54
Number of controls	54

Positive control mutant frequencies (4.0 $\mu\text{g/mL}$ 3-methylcholanthrene)

Mean ($\pm$ SD)	$567.0 \pm 248.3 \times 10^{-6}$
Range	218.9 to 1414.9×10^{-6}
Number of experiments	54
Number of controls	54

Because some experiments contained multiple controls, the number of independent control cultures exceeded the number of experiments.