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SUBTITLE: Evaluation of Five Unicharge Propellants in the In Vitro Sister Chromatid Exchange Assay in Chinese Hamster Ovary Cells

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n-Methyl-, n-ethyl- and n-butyl-2-nitratoethyl nitramine (MeNENA, EtNENA, and BuNENA), and bis- (2,2- dinitropropyl) acetal/formal (~50/50 mixture; ± diphenyl amine stabilizer; BDNPA/F±DPA), were tested in the in vitro SCE assay in CHO cells to a maximum of 5000 ug/ml or a toxic limit with and without a metabolic activation system (S-9 mix). EtNENA and BDNPA/F+DPA induced statistically significant, dose-related increases in SCE frequencies without S-9 mix, but only EtNENA induced two-fold increases (3.6-fold) over the negative control. MeNENA, EtNENA and BuNENA induced statistically significant, dose-related, 5.0- to 6.2-fold increases in SCE frequencies with S-9 mix. Both BDNPA/F±DPA induced statistically significant increases at several dose points, however, they were not two-fold increases at any dose. On the basis of these results, the rank order of SCE inducers is EtNENA>BuNENA>MeNENA>BDNPA/F+DPA=BDNPA/F-DPA.

14. SUBJECT TERMS

SCE/CHO assay, unicharge propellants, methyl-NENA, ethyl-NENA, butyl-NENA, bis-(2,2-dinitropropyl)acetal, bis-(2,2-dinitropropyl)formal, lab animals, RAIII

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SUMMARY

Five Unicharge propellant compounds were evaluated in the in vitro SCE Assay to determine their potential to induce an increase in SCE frequency over the solvent control, dimethyl sulfoxide (DMSO).

Selection of doses for the SCE assays were based upon preliminary cytotoxicity tests utilizing cell proliferation kinetics. This biological endpoint estimates the average proliferation time (APT) in which a population of CHO cells has undergone cell divisions in the presence of the thymidine analog, 5-bromo-2'-deoxyuridine (BrdUrd). Any increase in APT over the solvent control is an indication of cytotoxicity. All five test articles were evaluated for cytotoxicity in single cultures at doses of 5, 25, 50, 100, 250, 500, 750, 1000, 2500 and 5000 µg/ml with and without an exogenous metabolic activation system (S-9 mix). Based on the cytotoxicity findings, the following dose levels were selected to be evaluated in the SCE assay with and without S-9 mix for each of the five compounds to ensure five scorable doses: MeNENA was evaluated at 50, 100, 500, 1000, 2500 and 5000 µg/ml with and without S-9 mix. EtNENA was evaluated at doses of 50, 250, 500, 2000 and 5000 µg/ml without S-9 mix and at doses of 50, 250, 500, 2000, 4000 and 5000 µg/ml with S-9 mix. BuNENA was evaluated at doses of 10, 50, 150, 300, 500 and 600 µg/ml without S-9 mix and 10, 50, 150, 300, 400 and 500 µg/ml with S-9 mix. BDNPA/F+DPA was evaluated at doses of 1, 5, 15, 25, 40, 50, 60, 75, 100 and 150 µg/ml without S-9 mix and 1, 5, 15, 25, 30, 40, 50, 60, 75, 100 and 150 µg/ml with S-9 mix. BDNPA/F-DPA was evaluated at doses of 1, 5, 10, 15, 25, 40, 50, 75 and 100 µg/ml without S-9 mix and 1, 5, 10, 15, 25, 40, 50 and 60 µg/ml with S-9 mix.

In the SCE assay, duplicate cultures were established for each test point evaluated with and without S-9 mix. After a five hour treatment with each of the five test articles, the cultures were washed and fresh medium added. At this time, BrdUrd was added to each flask and cultures were incubated for an additional 29 hours. Two to three hours prior to harvest, colcemid (2×10^{-7}M) was added to each culture to arrest cells in metaphase. The cells were harvested and slides were prepared and stained for sister chromatid differentiation.

Prior to coding, slides were prescreened for toxicity and the following doses were coded for analysis: MeNENA was evaluated at doses of 50, 500, 1000, 2500 and 5000 µg/ml without S-9 mix and at doses of 50, 100, 500, 1000 and 2500 µg/ml with S-9 mix. EtNENA was evaluated at doses of 50, 250, 500, 2000 and 5000 µg/ml without

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SUMMARY

S-9 mix and at doses of 50, 250, 500, 2000 and 4000 µg/ml with S-9 mix. BuNENA was evaluated at doses of 10, 50, 150, 300 and 500 µg/ml with and without S-9 mix. BDNPA/F+DPA was evaluated at doses of 5, 25, 50, 75 and 100 µg/ml with and without S-9 mix. BDNPA/F-DPA was evaluated at doses of 5, 10, 25, 40 and 50 µg/ml with and without S-9 mix.

Statistical analyses of the data indicated that all five test articles induced statistically significant increases in SCE frequencies over the negative control, DMSO, with and/or without S-9 mix. Statistically significant, dose related increases in SCE frequencies with at least a two-fold increase over the negative control were observed for EtNENA without S-9 mix (3.6-fold increases) and for MeNENA, EtNENA and BuNENA with S-9 mix (5.1-6.2-fold increases) and therefore these increases were considered biologically significant. BDNPA/F±DPA did not induce two-fold increases at any of the dose levels scored.

Therefore, the results for the three NENA test articles were statistically and biologically positive, while BPNPA/F±DPA was only statistically positive in the SCE assay under the conditions, and according to the criteria, of the test protocol. On the basis of the results observed with S-9 mix, the rank order of SCE inducers was EtNENA > BuNENA > MeNENA > BPNPA/F+ = BPNPA/F-DPA.

STUDY DESCRIPTION

Sponsor:	U.S. Army Medical Research and Development Command Fort Detrick Frederick, Maryland 21702-5012
Study Numbers:	PH 319-US-001-91 PH 319-US-002-91 PH 319-US-003-91 PH 319-US-004-91 PH 319-US-005-91
Date Protocol Signed by Study Director:	September 23, 1991
Date Cytotoxicity Initiated:	October 2, 1991
Date Scoring of SCE Completed:	December 31, 1991
Sponsor's Study Monitor:	Major Nathaniel Powell, U.S. Army Medical Research and Development Laboratory
Pharmakon's Study Director:	Juan R. SanSebastian, Ph.D., Pharmakon Research International, Inc.
Technical Performance:	Juan R. SanSebastian, Ph.D., Patricia E. Giglio, B.S., Susan M. Lucenti, B.S., Mary Jo Berta, B.S., Ruth M. Sorg, M.S. (Hyg), Nancy Gongliewski and Nira Madison
Notebook Reference:	Notebook #1466, pages 118-150; 197-280

Good Laboratory Practices Statement: This study was conducted in compliance with the Good Laboratory Practice Regulations for non-clinical laboratory studies as developed by the U.S. Food and Drug Administration (Federal Register, Title 21, part 58), revised as of September 4, 1987, as well as the Organisation for Economic Co-operation and Development (OECD) Guidelines for Testing Chemicals, ISBN 92-64-12221-4, adopted by the council at its 535th meeting on 12 May, 1981, as well as the U.S. Environmental Protection Agency (EPA) as stated in the Federal Register, Title 40, Part 792, August 17, 1989.

Records Maintained: All correspondence pertinent to the study between the sponsor and Pharmakon Research International, Inc.

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including protocol, amendments to the protocol, raw data, test substance weight or volume, dispensation reports, quality assurance reports, and the final report, as well as microscope slides scored in the study, are maintained in the Pharmakon Archives.

PURPOSE

To evaluate the potential of the test article or its metabolites to induce SCE in CHO cells in culture.

Introduction: CHO-K1-BH4 cells, when grown in culture in the presence of the base analog 5-bromo-2'-deoxyuridine (BrdUrd) for two consecutive replication cycles, exhibit differential staining of their sister chromatids when stained with a Fluorescence-plus-Giemsa (FPG) staining technique (Perry and Wolff, 1974 and Goto et al., 1978). SCE are observed at the second mitosis as reciprocal alterations in staining pattern along the two chromatids of a chromosome. SCEs are widely accepted as sensitive indicators of mutagenic and/or carcinogenic potential (Perry and Evans, 1975 and Latt et al., 1980).

MATERIALS AND METHODS

Cell Line Designation: CHO-K1-BH4, Lot #M7
This is a continuous cell line with the modal number of 20 chromosomes with a population doubling time of 12-14 hours.

Source: Dr. Abraham W. Hsie
Biology Division
Oak Ridge National Laboratories
P.O. Box Y
Oak Ridge, Tennessee 37830

Subcloned by: Pharmakon Research International, Inc.

Test Articles: All five test articles were received by Pharmakon Research on September 19, 1991 in clear glass containers. n-methyl-2-nitratoethyl nitramine (MeNENA; CASE: 17096-47-8; Lot #XAP-MeNENA-6B), n-ethyl-2-nitratoethyl nitramine (EtNENA; CAS: 85068-73-1 Lot #XAP-EtNENA-4B) and N-butyl-2-nitratoethyl nitramine (BuNENA; CAS: 82486-82-6 Lot #XAP-BuNENA-15B) were provided as preweighed, single-use samples, and were described as a white solid, a yellow liquid, and a yellow liquid, respectively. MeNENA contained 30% added water for transport. Mixtures of bis-(2,2-dinitropropyl) acetal (CAS: 5108-69-0 and bis(2,2-dinitropropyl) formal (CAS: 5917-61-3), with and without diphenyl amine stabilizer (BDNPA/F±DPA), also were described as yellow liquids.

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Information regarding technical aspects of the test article, as provided by the sponsor, was recorded in the sponsor's file. For the purposes of this study, the test articles were stored at room temperature in the containers received from the sponsor. At the time of testing, the test articles exhibited the same physical characteristics as noted upon arrival. There was no apparent change in the physical states of the test articles during storage.

EtNENA, BuNENA, BDNPA/F +DPA, and BDNPA/F -DPA were used directly as received. However, samples of MeNENA were uncapped and placed in a desiccator (with desiccant) for approximately 24 hours in a Biological Safety Hood prior to use, to remove the added water. All required dilutions were made with dimethyl sulfoxide (DMSO), Lot #902873, supplied by Fisher Scientific (Fairlawn, NJ). Dilutions were prepared the day of the test and used less than two hours after preparation.

Control Articles: (1) Negative control was DMSO [Fisher Scientific, Lot #902873] for all five test articles at a final concentration of 1% (v/v).

(2) Positive Controls - The following known SCE inducer agents were selected:

(a) Ethylmethane Sulfonate (EMS) [Sigma, Lot #31H0701], a direct acting mutagen which does not require metabolic activation was the positive control for cultures without S-9 mix. EMS was dosed at a final treatment concentration of 124 μg/ml (10^{-3}M).

(b) N-nitrosodiethylamine (DEN), [Eastman Lot #A7A], a promutagen which requires metabolic activation was the positive control for the cultures treated with S-9 mix. DEN was dissolved in HPLC Grade Water and dispensed at 100 μl for a final treatment concentration of 100 μg/ml (9.8×10^{-4}M).

S-9 Metabolic Activation System: The S-9 activation mixture was prepared immediately prior to treatment. The S-9 mix contained (per ml) 10mM $MgCl_2$, 10mM $CaCl_2$, 30mM KCl, 5mM glucose-6-phosphate, 4mM NADP (disodium salt), 50mM sodium phosphate buffer (pH 7.4) and 0.1 ml of the microsomal preparation containing approximately 34.8 mg protein/ml. The microsomal preparation was obtained from Aroclor 1254 induced rat liver on January 23, 1991 and kept frozen at approximately -135°C in small aliquots (~2-3 ml).

Cytotoxicity: Single cultures of CHO-K1-BH4 cells were prepared at a density of 6×10^5 cells/80 cm^2 flask in F12FCM(5%) medium [Ham's medium F12 (K.C. Biological Co., reconstituted with deionized

water, adjusted to pH 7.5, followed by the addition of 1.2 g/l $NaHCO_3$) containing 5% heat-inactivated (56°C, 30 min.) fetal bovine serum (K.C. Biological Co.) extensively dialyzed by Pharmakon Research International, Inc.] Following the growth period, the medium was aspirated from each flask and fresh medium was added. Non-activated cultures were supplied with 10 ml of F12 serum free medium and activated cultures with 8 ml of F12 serum free medium and 2 ml of S-9 mixture. Treatment was initiated by the addition of 100 µl of each of the five test article or control dilutions to the appropriate cultures. All cultures were incubated for five hours at approximately 37°C in 5% CO_2 in air and 90+ % humidity. After treatment, cultures were washed three times with 5 ml of Saline-G, then 10 ml of fresh F12CM (5%) medium and BrdUrd (0.5×10^{-5}M final concentration) were dispensed to each flask. Flasks were incubated for an additional 28 hours at 37°C in 5% CO_2 in air and ≤90% humidity. For the last 2-3 hours of incubation, colcemid (2×10^{-7}M final concentration), was added to each culture to arrest cells in metaphase. At the end of incubation, cells were collected by the mitotic shake-off method and slides prepared and stained for sister chromatid differentiation (Terasima T. and Tolmach, L.J., 1961). All media and Saline-G were pre-warmed to 37°C prior to use.

It has been shown that an increase in osmolality (ion concentrations) and/or non-physiological pH are genotoxic to cultured mammalian cells (Brusick, D., 1986 and Galloway, et al., 1987 and Morita, T., et al., 1989). Therefore, the osmolality and pH of each of the test article dilutions were evaluated and compared to the negative control, DMSO (Tables 1-5).

CHO-SCE PROTOCOL

Preparation of Cells: Cells in logarithmic growth were detached with 0.05% trypsin solution and plated at a density of approximately 8×10^5 cells/80 cm^2 flask in 15 ml medium containing 5% heat inactivated calf bovine serum. Duplicate cultures were established for each control and treatment dose level both with and without S-9. Cells were then incubated at 37°C for approximately 16-24 hours.

Treatment: Following the growth period, the medium was aspirated from each flask and fresh medium was replaced. Non-activated cultures were supplied with 10 ml of F12 serum free medium and activated cultures with 8 ml of F12 serum free medium and 2 ml of S-9 mixture. Treatment was initiated by the addition of 100 µl of each of the five test article or control dilutions to the appropriate cultures. Cultures were incubated at 37°C in 5% CO_2 at 90+ % humidity for five hours.

Following treatment, cells were washed three times in 5 ml washes of Saline-G prewarmed at 37°C and supplied with 10 ml of medium F12FCM(5%) and 5µM BrdUrd. The cultures were incubated at 37°C, 5% CO_2 and 90+ % humidity for an additional 29 hours.

Cultures were incubated in the dark and exposed only to yellow safety light when necessary to avoid photolysis of BrdUrd-containing DNA (Ikushima and Wolff, 1974). For the last 2 hours of incubation, colcemid (2×10^{-7}M final concentration) was added to each culture to arrest cells in metaphase.

Slide Preparation: At the end of incubation, cell suspensions were collected by the mitotic shake-off method. Cells were sedimented by centrifugation for approximately 5-10 minutes at 1000 rpm and hypotonic KCl (0.075M) added to swell the cells. Cells were fixed in three washes of methanol:glacial acetic acid (3 parts: 1 part) and slides prepared by standard methods. Staining of slides by the FPG method included: 1.0 µg/ml Hoechst 33258 stain, black light irradiation and 2-3% Giemsa stain. Slides were air-dried and coverslips mounted.

DATA ANALYSIS

Coding of Slides: Slides were coded randomly by study number, and each duplicate culture was assigned a separate code number. Slides were coded as a single experiment without regard to the presence or absence of S-9 mix by a person not involved in the actual scoring of the study.

Slide Analysis: Generally, a total of 50 (25 metaphases per culture) well-spread, second division cells containing ± 2 centromeres from the modal number of 20 were scored for each dose level. SCE were scored as reciprocal alterations in staining pattern along the chromatids of a chromosome. Cells were counted for chromosome number and data are presented as SCE/metaphase and SCE/chromosome. The mean cell cycle (MCC) is based on the ratio of first, second and third division metaphases per metaphases scored. The APT is expressed as the ratio of exposure time of a population of cells in BrdUrd to the respective MCC.

Evaluation Criteria: Assessment of a test article as positive is based upon its ability to produce a statistically significant increase in the SCE frequency as compared to the concurrent solvent control. If the *t* test indicates a statistically positive result at a single dose level only, this is insufficient grounds to regard the test article as positive, although the presence of a dose response in consecutive dose levels will justify retesting, using additional concentrations and/or fixation times (Perry et al., 1984).

Whatever the statistical approach, SCE results should be interpreted with due regard for the biological significance of the data. For biological significance, there should be a two-fold increase in SCE frequency in at least one dose level as compared to the negative control and/or a significant dose-response pattern.

Statistical Analyses: Data for each test article were compared separately. Duplicate cultures were pooled to make a total of 50 scored metaphases per dose level. The SCE/metaphase data was transformed by a standard square root transformation. A t test on the transformed data compared each dose level against the solvent control.

Criteria for a Valid Assay: To be valid, the negative control, DMSO, must have $\leq$ 18 SCE per metaphase and the positive control must show a significant ($p \geq 0.05$) increase in SCE frequency as compared to the negative control.

RESULTS AND DISCUSSION

Five unicharge propellant compounds were evaluated in the In Vitro SCE assay to determine their potential to induce an increase in SCE frequency as compared to DMSO, the solvent control, in CHO cells with and without S-9 mix. Cytotoxicity of each compound was first evaluated utilizing cell proliferation kinetics as a parameter. All five test articles were evaluated at doses of 5, 25, 50, 100, 250, 500, 750, 1000, 2500 and 5000 µg/ml with and without S-9 mix. Based on the cytotoxicity findings, all five test articles were then evaluated with the appropriate doses in the SCE assay in duplicate cultures in the presence and absence of S-9 mix.

Fifty metaphases (25 metaphases per culture) were scored for sister chromatid exchange. SCE/metaphase data was tabulated and transformed by a standard square root transformation. A t test on the transformed data compared each dose level against the solvent control. Results of the SCE analyses and cell proliferation kinetics analyses are found in Tables 11a-15b (pages 27-36).

Both positive controls, EMS (10^{-3}M) and DEN (9.8×10^{-4}) produced significant increases in SCE frequency as compared to their respective solvent controls. The positive finding of DEN indicates that the S-9 activation system was functioning biologically. The positive response of the control article (EMS and DEN) demonstrates the integrity of the study.

MeNENA

n-methyl-2-nitratoethyl nitramine (MeNENA) was initially evaluated in a cytotoxicity test in CHO cells at doses of 5, 25, 50, 100, 250, 500, 750, 1000, 2500 and 5000 µg/ml with and without S-9 mix. The results of this assay (Table 6) indicate there was no significant increase in average proliferation time (APT) at any dose evaluated without S-9 mix. However, MeNENA produced a slightly toxic effect at the two highest doses with S-9 mix, indicated by a 35% and 32% increase in APT, respectively. Based on these findings, MeNENA was evaluated in the SCE assay at doses of 50, 100, 500, 1000, 2500 and 5000 µg/ml with and without S-9 mix to ensure five scorable doses. Prior to coding, the slides were screened and there were enough scorable metaphases in M_2 at all dose levels evaluated except for 5000 µg/ml with S-9 mix. Therefore, the following doses were coded for analysis: 50, 500, 1000, 2500 and 5000 µg/ml without S-9 mix and at 50, 100, 500, 1000

and 2500 µg/ml with S-9 mix. MeNENA, in the absence of S-9, induced a statistically significant increase ($p \leq 0.05$) in SCE frequency only at the high dose, 5000 µg/ml. However, this increase was not two-fold over the negative control which was the criterion for biological significance. MeNENA produced dose-dependent increases in the frequency of SCE at all doses evaluated with S-9 mix, with a five-fold increase over the negative control at the high dose, 2500 µg/ml. These results along with the cell proliferation kinetics analysis for the SCE assay are presented on Tables 11a, b and Figures 1a, b.

EtNENA

N-ethyl-2-2 nitratethyl nitramine (EtNENA) was initially evaluated in a cytotoxicity test in CHO cells at doses of 5, 25, 50, 100, 250, 500, 750, 1000, 2500 and 5000 µg/ml with and without S-9 mix. The results of this assay (Table 7) indicate there was no significant increase in APT at any dose evaluated without S-9 mix. However, EtNENA produced a significant dose-related increase in APT (50%) with S-9 mix at the highest dose (5000 µg/ml). Based on these findings, EtNENA was evaluated in the SCE assay at doses of 50, 250, 500, 2000 and 5000 µg/ml without S-9 mix and at doses of 50, 250, 500, 2000, 4000 and 5000 µg/ml with S-9 mix to ensure five scorable doses. At the time of colcemid addition, the majority of the cells in the 5000 µg/ml cultures with S-9 mix were shrunken, detached and lysed. These cultures were discarded. Therefore, the remaining doses with and without S-9 mix were coded for SCE analysis. EtNENA induced statistically significant, dose-related increases in SCE frequencies at all doses evaluated with approximately 3.6- to 6.3-fold increases over the negative control, DMSO, with and without S-9 mix, respectively, except for the 50 µg/ml dose without S-9 mix. These results along with the cell proliferation kinetics analysis for the SCE assay are presented on Tables 12a, b and Figures 2a, b.

BuNENA

N-butyl-2-nitraethyl nitramine (BuNENA) was initially evaluated in a cytotoxicity test in CHO cells at doses of 5, 25, 50, 100, 250, 500, 750, 1000, 2500 and 5000 µg/ml with and without S-9 mix. The results of this assay (Table 8) indicate BuNENA was cytotoxic to CHO cells from 750 µg/ml to 5000 µg/ml with and without S-9 mix. BuNENA was not toxic up to 500 µg/ml without S-9 mix and slightly toxic at 500 µg/ml (28% increase in APT) with S-9 mix. Based on these findings, BuNENA was evaluated in the SCE assay at doses of 10, 50, 150, 300, 500 and 600 µg/ml without S-9 mix and 10, 50, 150, 300, 400 and 500 µg/ml with S-9 mix. Prior to coding, the slides were prescreened for toxicity and there were enough scorable metaphases in M_2 at all doses evaluated except for the 600 µg/ml dose level without S-9 mix. Therefore, the following doses were coded for SCE analysis: 10, 50, 150, 300 and 500 µg/ml with and without S-9 mix. BuNENA induced statistically significant, dose-related increases in SCE frequencies, in all doses with S-9 mix and in all doses except 50 µg/ml without S-9 mix with approximately a five-fold increase over the negative control, DMSO, with S-9 mix. BuNENA apparently reached a plateau at the two highest doses with S-9 mix, probably due to the selective killing of the severely

damaged cells, as was observed by the number of chromosomal aberrations in the M_1 metaphases. These results along with the cell proliferation kinetics analysis for the SCE assay are presented on Tables 13a, b and Figures 3a, b.

BDNPA/F+DPA

Bis-(2,2-dinitropropyl) acetal/formal with DPA stabilizer (BDNPA/F +DPA) was initially evaluated in a cytotoxicity test in CHO cells at doses of 5, 25, 50, 100, 250, 500, 750, 1000, 2500 and 5000 µg/ml with and without S-9 mix. The results of the assay (Table 9) indicated BDNPA/F+DPA was toxic to CHO cells from 250 to 5000 µg/ml with and without S-9 mix. However, the next lower dose, 100 µg/ml was not toxic. Based on these findings, BDNPA/F+DPA was evaluated in the SCE assay at doses of 1, 5, 25, 50, 100 and 150 µg/ml to achieve the highest possible scorable dose and at the same time ensure five scorable doses. At the time of colcemid addition, the majority of the cells in the 150 µg/ml cultures with and without S-9 mix were rounded up, floating and/or lysed. These cultures were discarded. Prior to coding the rest of the cultures, slides were prescreened for the quality and number of scorable metaphases in M_2. Due to technical problems at some dose levels in which there were not enough scorable metaphases, BDNPA/F+DPA was re-evaluated with some extra doses under the same conditions. Slides were prepared and the following doses were coded for SCE analysis from both experiments: 5, 25, 50, 75 and 100 µg/ml with and without S-9 mix, including both sets of negative controls (DMSO-1 and DMSO-2). BDNPA/F +DPA induced statistically significant, increases in SCE frequencies ($p \leq 0.01$) with and without S-9 mix and was dose related without S-9 mix. These increases were not two-fold, therefore, they were not considered biologically significant. These results, along with the cell proliferation kinetics analyses for the SCE assay are presented on Tables 14a, b and Figures 4a, b.

DNPA/F-DPA

Bis-(2,2-dinitropropyl) acetal/formal without DPA stabilizer (BDNPA/F-DPA) was initially evaluated in a cytotoxicity test in CHO cells at doses of 5, 25, 50, 100, 250, 500, 750, 1000, 2500 and 5000 µg/ml with and without S-9 mix. The results of the assay (Table 10) indicated BDNPA/F-DPA was toxic to CHO cells from 250 µg/ml to 5000 µg/ml without S-9 mix and from 100 µg/ml to 5000 µg/ml with S-9 mix. BDNPA/F-DPA induced a dose-related increase in APT from 25 µg/ml to 100 µg/ml without S-9 mix and from 5 µg/ml to 50 µg/ml with S-9 mix. The maximum increases in APT were 35% and 42% with and without S-9 mix, respectively. Based on these findings, BDNPA/F-DPA was evaluated in the SCE assay at doses of 5, 10, 25, 50, 75 and 100 µg/ml without S-9 mix and at doses of 1, 5, 10, 25, 40, 50 and 60 µg/ml with S-9 mix to ensure five scorable doses. At the time of colcemid addition, it was observed that the majority of the cells from the 75 µg/ml and 100 µg/ml cultures were shrunken and floating, therefore, these cultures were not

harvested. Slides were prepared from the remaining cultures. Prior to coding, the slides were prescreened for quality and number of scorable metaphases in M_2. It was observed that the 60 µg/ml cultures with S-9 mix had no scorable metaphases. Therefore BDNPA/F-DPA was re-evaluated under similar conditions. Slides were prepared and this time all dose levels evaluated had sufficient scorable metaphases in M_2. The following doses were coded for SCE analysis: 5, 10, 25, 40 and 50 µg/ml with and without S-9 mix. BNDPA/F-DPA, in the absence of S-9 mix, did not induce a statistically significant increase at any of the doses analyzed. However, BNDPA/F-DPA, in the presence of S-9 mix, produced a statistically significant increase ($p \geq 0.01$) at the two highest doses analyzed, but these increases were not two-fold over the control values. These results along with the cell proliferation kinetics analysis for the SCE assay are presented on Tables 15a, b and Figures 5a, b. To verify the biological significance of the statistical findings for BDNPA/F±DPA, we suggest an independent retest.

Osmolality

There were no significant changes in the pH and/or osmolality of the dosing solutions as compared to the respective solvent controls (Tables 1-5). However, the solvent control DMSO, had a significant increase in osmolality over the untreated control (F12 medium), but did not induce an increase in the SCE frequency as compared to the solvent control (Tables 11a-15a).

CONCLUSIONS

All five test articles induced statistically significant increases in SCE frequencies as compared to the solvent control with and/or without S-9 mix. Statistically significant, dose related increases in SCE frequencies with at least a two-fold increase over the negative control were observed for EtNENA without S-9 mix (3.6-fold increases) and for MeNENA, EtNENA and BuNENA with S-9 mix (5.1-6.2-fold increases). Therefore, the three NENA test articles were statistically and biologically significant in increasing SCE frequencies over the negative controls DMSO. However, BDNPA/F±DPA did not induce two-fold increases in SCE frequency at any of the dose levels scored. A summary of SCE results are presented in Table 16.

In conclusion, the three NENA test articles were statistically and biologically positive and BDNPA/F±DPA were only statistically positive under the conditions, and according to the criteria, of the test protocol. On the basis of the results observed with S-9 mix, the rank of order of mutagenic potential is EtNENA > BuNENA > MeNENA > BDNPA/F+DPA = BDNPA/F-DPA.

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(GT/RP13(319US011)

Table 1 - Osmolality and pH in Culture Medium of CHO cells

Compound	Dose µg/ml	S-9	Phase	Osmolality (mOSM/kg H_2O) $\bar{x}$	pH
Untreated	0	-	At-treatment	288	8.08
DMSO (1%)	0	-	At-treatment	437	8.88
MeNENA	5	-	At-treatment	443	9.05
MeNENA	25	-	At-treatment	434	9.09
MeNENA	50	-	At-treatment	440	9.09
MeNENA	100	-	At-treatment	441	9.11
MeNENA	250	-	At-treatment	429	9.11
MeNENA	500	-	At-treatment	431	9.05
MeNENA	750	-	At-treatment	430	8.98
MeNENA	1000	-	At-treatment	397	8.97
MeNENA	2500	-	At-treatment	441	8.96
MeNENA	5000	-	At-treatment	403	8.95
Untreated	0	-	Post-treatment	NA	7.09
DMSO (1%)	0	-	Post-treatment	NA	7.15
MeNENA	5	-	Post-treatment	NA	7.34
MeNENA	25	-	Post-treatment	NA	7.43
MeNENA	50	-	Post-treatment	NA	7.53
MeNENA	100	-	Post-treatment	NA	7.54
MeNENA	250	-	Post-treatment	NA	7.55
MeNENA	500	-	Post-treatment	NA	7.54
MeNENA	750	-	Post-treatment	NA	7.58
MeNENA	1000	-	Post-treatment	NA	7.60
MeNENA	2500	-	Post-treatment	NA	7.59
MeNENA	5000	-	Post-treatment	NA	7.65

NA - Not applicable

NOTE: The S-9 mix was added to a set of cultures to test the potential of the test article to be biotransformed by the liver enzymes (cytochrome P-450) into a clastogen. Consequently, the osmolality and pH of the solvent and dosing solutions were only measured in the set of cultures without S-9 mix. The high pH at treatment was due to the buffer capacity (sodium bicarbonate) of the F12SF medium. Once the cultures were placed in 5% CO_2 incubator, the pH of the medium became equilibrated to a physiologic pH. Therefore, the pH was measured at-and post-treatment times while the osmolality was only measured at treatment time.

Table 2 - Osmolality and pH in Culture Medium of CHO cells

Compound	Dose µg/ml	S-9	Phase	Osmolality (mOSM/kg H_2O) $\bar{x}$	pH
Untreated	0	-	At-treatment	297	8.56
DMSO (1%)	0	-	At-treatment	441	8.60
EtNENA	5	-	At-treatment	400	8.61
EtNENA	25	-	At-treatment	340	8.66
EtNENA	50	-	At-treatment	461	8.66
EtNENA	100	-	At-treatment	439	8.64
EtNENA	250	-	At-treatment	403	8.65
EtNENA	500	-	At-treatment	427	8.65
EtNENA	750	-	At-treatment	416	8.68
EtNENA	1000	-	At-treatment	426	8.68
EtNENA	2500	-	At-treatment	410	8.67
EtNENA	5000	-	At-treatment	407	8.70
Untreated	0	-	Post-treatment	NA	6.93
DMSO (1%)	0	-	Post-treatment	NA	6.99
EtNENA	5	-	Post-treatment	NA	7.09
EtNENA	25	-	Post-treatment	NA	7.10
EtNENA	50	-	Post-treatment	NA	7.18
EtNENA	100	-	Post-treatment	NA	7.19
EtNENA	250	-	Post-treatment	NA	7.19
EtNENA	500	-	Post-treatment	NA	7.23
EtNENA	750	-	Post-treatment	NA	7.20
EtNENA	1000	-	Post-treatment	NA	7.19
EtNENA	2500	-	Post-treatment	NA	7.26
EtNENA	5000	-	Post-treatment	NA	7.19

NA - Not applicable

NOTE: The S-9 mix was added to a set of cultures to test the potential of the test article to be biotransformed by the liver enzymes (cytochrome P-450) into a clastogen. Consequently, the osmolality and pH of the solvent and dosing solutions were only measured in the set of cultures without S-9 mix. The high pH at treatment was due to the buffer capacity (sodium bicarbonate) of the F12SF medium. Once the cultures were placed in 5% CO_2 incubator, the pH of the medium became equilibrated to a physiologic pH. Therefore, the pH was measured at-and post-treatment times while the osmolality was only measured at treatment time.

Table 3 - Osmolality and pH in Culture Medium of CHO cells

Compound	Dose µg/ml	S-9	Phase	Osmolality (mOSM/kg H_2O) $\bar{x}$	pH
Untreated	0	-	At-treatment	261	8.94
DMSO (1%)	0	-	At-treatment	399	8.98
BuNENA	5	-	At-treatment	399	9.00
BuNENA	25	-	At-treatment	379	9.02
BuNENA	50	-	At-treatment	368	9.01
BuNENA	100	-	At-treatment	321	9.01
BuNENA	250	-	At-treatment	375	9.01
BuNENA	500	-	At-treatment	365	9.01
BuNENA	750	-	At-treatment	367	9.00
BuNENA	1000	-	At-treatment	380	9.01
BuNENA	2500	-	At-treatment	326	9.01
BuNENA	5000	-	At-treatment	318	9.00
Untreated	0	-	Post-treatment	NA	-[a]
DMSO (1%)	0	-	Post-treatment	NA	-
BuNENA	5	-	Post-treatment	NA	-
BuNENA	25	-	Post-treatment	NA	-
BuNENA	50	-	Post-treatment	NA	-
BuNENA	100	-	Post-treatment	NA	-
BuNENA	250	-	Post-treatment	NA	-
BuNENA	500	-	Post-treatment	NA	-
BuNENA	750	-	Post-treatment	NA	-
BuNENA	1000	-	Post-treatment	NA	-
BuNENA	2500	-	Post-treatment	NA	-
BuNENA	5000	-	Post-treatment	NA	-

NA - Not applicable

[a]Inadvertently discarded post treatment medium; no pH values determined.

NOTE: The S-9 mix was added to a set of cultures to test the potential of the test article to be biotransformed by the liver enzymes (cytochrome P-450) into a clastogen. Consequently, the osmolality and pH of the solvent and dosing solutions were only measured in the set of cultures without S-9 mix. The high pH at treatment was due to the buffer capacity (sodium bicarbonate) of the F12SF medium. Once the cultures were placed in 5% CO_2 incubator, the pH of the medium became equilibrated to a physiologic pH. Therefore, the pH was measured at-and post-treatment times while the osmolality was only measured at treatment time.

Table 4 - Osmolality and pH in Culture Medium of CHO cells

Compound	Dose µg/ml	S-9	Phase	Osmolality (mOSM/kg H_2O) $\bar{x}$	pH
Untreated	0	-	At-treatment	287	8.77
DMSO (1%)	0	-	At-treatment	422	9.00
BDNPA/F +DPA	5	-	At-treatment	438	9.04
BDNPA/F +DPA	25	-	At-treatment	445	9.05
BDNPA/F +DPA	50	-	At-treatment	456	9.06
BDNPA/F +DPA	100	-	At-treatment	455	9.07
BDNPA/F +DPA	250	-	At-treatment	448	9.07
BDNPA/F +DPA	500	-	At-treatment	443	9.07
BDNPA/F +DPA	750	-	At-treatment	439	9.07
BDNPA/F +DPA	1000	-	At-treatment	444	9.07
BDNPA/F +DPA	2500	-	At-treatment	430	9.08
BDNPA/F +DPA	5000	-	At-treatment	404	9.08
Untreated	0	-	Post-treatment	NA	7.13
DMSO (1%)	0	-	Post-treatment	NA	7.32
BDNPA/F +DPA	5	-	Post-treatment	NA	7.48
BDNPA/F +DPA	25	-	Post-treatment	NA	7.58
BDNPA/F +DPA	50	-	Post-treatment	NA	7.64
BDNPA/F +DPA	100	-	Post-treatment	NA	7.65
BDNPA/F +DPA	250	-	Post-treatment	NA	7.69
BDNPA/F +DPA	500	-	Post-treatment	NA	7.74
BDNPA/F +DPA	750	-	Post-treatment	NA	7.74
BDNPA/F +DPA	1000	-	Post-treatment	NA	7.74
BDNPA/F +DPA	2500	-	Post-treatment	NA	7.75
BDNPA/F +DPA	5000	-	Post-treatment	NA	7.77

NA - Not applicable

NOTE: The S-9 mix was added to a set of cultures to test the potential of the test article to be biotransformed by the liver enzymes (cytochrome P-450) into a clastogen. Consequently, the osmolality and pH of the solvent and dosing solutions were only measured in the set of cultures without S-9 mix. The high pH at treatment was due to the buffer capacity (sodium bicarbonate) of the F12SF medium. Once the cultures were placed in 5% CO_2 incubator, the pH of the medium became equilibrated to a physiologic pH. Therefore, the pH was measured at-and post-treatment times while the osmolality was only measured at treatment time.

Table 5 - Osmolality and pH in Culture Medium of CHO cells

Compound	Dose µg/ml	S-9	Phase	Osmolality (mOSM/kg H_2O) $\bar{x}$	pH
Untreated	0	-	At-treatment	242	8.67
DMSO (1%)	0	-	At-treatment	430	8.89
BDNPA/F -DPA	5	-	At-treatment	442	8.95
BDNPA/F -DPA	25	-	At-treatment	444	8.97
BDNPA/F -DPA	50	-	At-treatment	436	8.98
BDNPA/F -DPA	100	-	At-treatment	441	8.99
BDNPA/F -DPA	250	-	At-treatment	432	9.00
BDNPA/F -DPA	500	-	At-treatment	436	8.99
BDNPA/F -DPA	750	-	At-treatment	425	9.00
BDNPA/F -DPA	1000	-	At-treatment	436	9.00
BDNPA/F -DPA	2500	-	At-treatment	424	9.01
BDNPA/F -DPA	5000	-	At-treatment	399	9.01
Untreated	0	-	Post-treatment	NA	7.15
DMSO (1%)	0	-	Post-treatment	NA	7.29
BDNPA/F -DPA	5	-	Post-treatment	NA	7.44
BDNPA/F -DPA	25	-	Post-treatment	NA	7.47
BDNPA/F -DPA	50	-	Post-treatment	NA	7.43
BDNPA/F -DPA	100	-	Post-treatment	NA	7.46
BDNPA/F -DPA	250	-	Post-treatment	NA	7.48
BDNPA/F -DPA	500	-	Post-treatment	NA	7.49
BDNPA/F -DPA	750	-	Post-treatment	NA	7.48
BDNPA/F -DPA	1000	-	Post-treatment	NA	7.53
BDNPA/F -DPA	2500	-	Post-treatment	NA	7.59
BDNPA/F -DPA	5000	-	Post-treatment	NA	7.60

NA - Not applicable

NOTE: The S-9 mix was added to a set of cultures to test the potential of the test article to be biotransformed by the liver enzymes (cytochrome P-450) into a clastogen. Consequently, the osmolality and pH of the solvent and dosing solutions were only measured in the set of cultures without S-9 mix. The high pH at treatment was due to the buffer capacity (sodium bicarbonate) of the F12SF medium. Once the cultures were placed in 5% CO_2 incubator, the pH of the medium became equilibrated to a physiologic pH. Therefore, the pH was measured at-and post-treatment times while the osmolality was only measured at treatment time.

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Table 6 - Proliferation Kinetics Analysis - Cytotoxicity

Compound	Dose (µg/ml)	S-9 (±)	Total No. Metaphases Scored	No. of Mitotic Divisions M1	M1+	M2	M2+	M3	MCC	APT (hrs)	%APT Increase[1]
Untreated	0	-	100	0	0	88	12	0	2.06	13.59	0.00
DMSO (1%)	0	-	100	1	3	85	11	0	2.03	13.79	-
MeNENA	5	-	100	0	3	91	6	0	2.02	13.86	0.51
MeNENA	25	-	100	2	15	78	5	0	1.93	14.51	5.22
MeNENA	50	-	100	2	14	83	1	0	1.92	14.58	5.73
MeNENA	100	-	100	5	26	68	1	0	1.83	15.30	10.95
MeNENA	250	-	100	4	23	71	2	0	1.86	15.05	9.14
MeNENA	500	-	100	4	13	82	1	0	1.90	14.74	6.89
MeNENA	750	-	100	8	24	66	2	0	1.81	15.47	12.18
MeNENA	1000	-	100	4	14	78	4	0	1.91	14.66	6.31
MeNENA	2500	-	100	0	5	92	3	0	1.99	14.07	2.03
MeNENA	5000	-	100	2	22	76	0	0	1.87	14.97	8.56
Untreated	0	+	100	0	3	73	24	0	2.11	13.27	0.00
DMSO (1%)	0	+	100	0	5	75	20	0	2.08	13.46	-
MeNENA	5	+	100	0	13	81	6	0	1.97	14.21	5.57
MeNENA	25	+	100	3	25	72	0	0	1.85	15.14	12.48
MeNENA	50	+	100	2	23	73	2	0	1.88	14.89	10.62
MeNENA	100	+	100	3	40	57	0	0	1.77	15.82	17.53
MeNENA	250	+	100	4	40	56	0	0	1.76	15.91	18.20
MeNENA	500	+	100	6	49	45	0	0	1.70	16.47	22.36
MeNENA	750	+	100	2	25	73	0	0	1.86	15.05	11.81
MeNENA	1000	+	100	0	33	67	0	0	1.84	15.22	13.08
MeNENA	2500	+	100	18	56	26	0	0	1.54	18.18	35.07
MeNENA	5000	+	100	13	58	29	0	0	1.58	17.72	31.65

Time in BrdUrd = 28 Hours.

1 - % APT increase is based on a comparison of each dose level to the solvent control.

See page 43 for Legend to Cell Proliferation Kinetics.

PH 319-US-002-91

Table 7 - Cell Proliferation Kinetics Analysis - Cytotoxicity

Compound	Dose (μg/ml)	S-9 (±)	Total No. Metaphases Scored	No. of Mitotic Divisions M1	M1+	M2	M2+	M3	MCC	APT (hrs)	%APT Increase[1]
Untreated	0	-	100	1	24	75	0	0	1.87	14.97	4.25
DMSO (1%)	0	-	100	1	10	88	1	0	1.95	14.36	-
EtNENA	5	-	100	1	11	86	2	0	1.95	14.36	0.00
EtNENA	25	-	100	0	14	82	4	0	1.95	14.36	0.00
EtNENA	50	-	100	2	8	85	5	0	1.97	14.21	0.00
EtNENA	100	-	100	3	7	88	2	0	1.95	14.36	0.00
EtNENA	250	-	100	3	10	85	2	0	1.93	14.51	1.04
EtNENA	500	-	100	0	15	76	9	0	1.97	14.21	0.00
EtNENA	750	-	100	5	15	78	2	0	1.89	14.81	3.13
EtNENA	1000	-	100	1	10	88	1	0	1.95	14.36	0.00
EtNENA	2500	-	100	2	5	91	2	0	1.97	14.21	0.00
EtNENA	5000	-	100	5	12	83	0	0	1.89	14.81	3.13
Untreated	0	+	100	1	5	93	1	0	1.97	14.21	0.50
DMSO (1%)	0	+	100	0	9	87	4	0	1.98	14.14	-
EtNENA	5	+	100	0	14	84	2	0	1.94	14.43	2.05
EtNENA	25	+	100	5	17	78	0	0	1.87	14.97	5.87
EtNENA	50	+	100	7	22	71	0	0	1.82	15.38	8.77
EtNENA	100	+	100	4	31	65	0	0	1.81	15.47	9.41
EtNENA	250	+	100	15	37	43	0	0	1.67	16.77	18.60
EtNENA	500	+	100	9	45	46	0	0	1.69	16.57	17.19
EtNENA	750	+	100	10	44	46	0	0	1.68	16.67	17.89
EtNENA	1000	+	100	5	59	36	0	0	1.66	16.87	19.31
EtNENA	2500	+	100	28	63	9	0	0	1.41	19.86	40.45[2]
EtNENA	5000	+	100	42	53	5	0	0	1.32	21.21	50.00[2]

Time in BrdUrd = 28 Hours.

1 - % APT increase is based on a comparison of each dose level to the solvent control. See page 43 for Legend to Cell Proliferation Kinetics.

2 - 40 and 50% increases indicated a significant increases in APT. Generally the highest dose selected for SCE is the dose which increase APT ≤50%.

PH 319-US-003-91

Table 8 - Cell Proliferation Kinetics Analysis - Cytotoxicity

Compound	Dose (μg/ml)	S-9 (±)	Total No. Metaphases Scored	No. of Mitotic Divisions M1	M1+	M2	M2+	M3	MCC	APT (hrs)	%APT Increase[1]
Untreated	0	-	100	2	5	85	8	0	2.00	14.00	0.00
DMSO (1%)	0	-	100	1	6	90	3	0	1.98	14.14	-
BuNENA	5	-	100	3	8	87	2	0	1.94	14.43	2.05
BuNENA	25	-	100	3	5	85	7	0	1.98	14.14	0.00
BuNENA	50	-	100	1	3	87	9	0	2.02	13.86	0.00
BuNENA	100	-	100	3	1	91	5	0	1.99	14.07	0.00
BuNENA	250	-	100	2	3	90	5	0	1.99	14.07	0.00
BuNENA	500	-	100	3	18	67	12	0	1.94	14.43	2.05
BuNENA	750	-	NH*								
BuNENA	1000	-	NH								
BuNENA	2500	-	NH								
BuNENA	5000	-	NH								
Untreated	0	+	100	3	3	81	13	0	2.02	13.86	0.00
DMSO (1%)	0	+	100	1	10	80	9	0	1.99	14.07	-
BuNENA	5	+	100	2	13	79	6	0	1.95	14.36	2.06
BuNENA	25	+	100	3	8	81	8	0	1.97	14.21	1.00
BuNENA	50	+	100	3	12	82	3	0	1.93	14.51	3.13
BuNENA	100	+	100	0	14	80	6	0	1.96	14.29	1.56
BuNENA	250	+	100	8	29	63	0	0	1.78	15.73	11.80
BuNENA	500	+	100	24	41	35	0	0	1.56	17.95	27.58
BuNENA	750	+	NH								
BuNENA	1000	+	NH								
BuNENA	2500	+	NH								
BuNENA	5000	+	NH								

Time in BrdUrd = 28 Hours.

1 - % APT increase is based on a comparison of each dose level to the solvent control.

See page 37 for Legend to Cell Proliferation Kinetics.

*NH - Not harvested; no cell survival.

PH 319-US-004-91

Table 9 - Cell Proliferation Kinetics Analysis - Cytotoxicity

Compound	Dose (μg/ml)	S-9 (±)	Total No. Metaphases Scored	No. of Mitotic Divisions M1	M1+	M2	M2+	M3	MCC	APT (hrs)	%APT Increase[1]
Untreated	0	-	100	3	0	86	11	0	2.03	13.79	0.00
DMSO (1%)	0	-	100	1	4	88	7	0	2.01	13.93	-
BDNPA/F +DPA	5	-	100	0	3	91	6	0	2.02	13.86	0.00
BDNPA/F +DPA	25	-	100	4	15	76	5	0	1.91	14.66	5.24
BDNPA/F +DPA	50	-	100	4	46	47	3	0	1.75	16.00	14.86
BDNPA/F +DPA	100	-	100	3	7	85	5	0	1.96	14.29	2.58
BDNPA/F +DPA	250	-	NH*								
BDNPA/F +DPA	500	-	NH								
BDNPA/F +DPA	750	-	NH								
BDNPA/F +DPA	1000	-	NH								
BDNPA/F +DPA	2500	-	NH								
BDNPA/F +DPA	5000	-	NH								
Untreated	0	+	100	1	4	66	29	0	2.12	13.21	0.00
DMSO (1%)	0	+	100	1	3	79	17	0	2.06	13.59	-
BDNPA/F +DPA	5	+	100	0	7	76	17	0	2.05	13.66	0.52
BDNPA/F +DPA	25	+	100	3	44	52	1	0	1.76	15.91	17.07
BDNPA/F +DPA	50	+	100	5	16	74	5	0	1.90	14.74	8.46
BDNPA/F +DPA	100	+	100	0	7	91	2	0	1.98	14.14	4.05
BDNPA/F +DPA	250	+	NH								
BDNPA/F +DPA	500	+	NH								
BDNPA/F +DPA	750	+	NH								
BDNPA/F +DPA	1000	+	NH								
BDNPA/F +DPA	2500	+	NH								
BDNPA/F +DPA	5000	+	NH								

Time in BrdUrd = 28 Hours. *NH - not harvested; no cell survival.

1 - % APT increase is based on a comparison of each dose level to the solvent control.

See page 43 for Legend to Cell Proliferation Kinetics.

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Table 10 - Cell Proliferation Kinetics Analysis - Cytotoxicity

Compound	Dose (μg/ml)	S-9 (±)	Total No. Metaphases Scored	No. of Mitotic Divisions M1	M1+	M2	M2+	M3	MCC	APT (hrs)	%APT Increase[1]
Untreated	0	-	100	2	6	91	1	0	1.96	14.29	0.00
DMSO (1%)	0	-	100	3	4	92	1	0	1.96	14.29	-
BDNPA/F -DPA	5	-	100	1	12	82	5	0	1.96	14.29	0.00
BDNPA/F -DPA	25	-	100	2	17	80	1	0	1.90	14.74	3.15
BDNPA/F -DPA	50	-	100	23	48	29	0	0	1.53	18.30	28.06
BDNPA/F -DPA	100	-	100	42	41	16	1	0	1.38	20.29	41.99
BDNPA/F -DPA	250	-	NH*								
BDNPA/F -DPA	500	-	NH								
BDNPA/F -DPA	750	-	NH								
BDNPA/F -DPA	1000	-	NH								
BDNPA/F -DPA	2500	-	NH								
BDNPA/F -DPA	5000	-	NH								
Untreated	0	+	100	0	2	89	9	0	2.04	13.73	0.00
DMSO (1%)	0	+	100	0	4	90	6	0	2.01	13.93	-
BDNPA/F -DPA	5	+	100	0	10	88	2	0	1.96	14.29	2.58
BDNPA/F -DPA	25	+	100	0	16	84	0	0	1.92	14.58	4.67
BDNPA/F -DPA	50	+	100	39	25	36	0	0	1.49	18.79	34.89
BDNPA/F -DPA	100	+	NH								
BDNPA/F -DPA	250	+	NH								
BDNPA/F -DPA	500	+	NH								
BDNPA/F -DPA	750	+	NH								
BDNPA/F -DPA	1000	+	NH								
BDNPA/F -DPA	2500	+	NH								
BDNPA/F -DPA	5000	+	NH								

Time in BrdUrd = 28 Hours. *NH - not harvested; no cell survival.

1 - % APT increase is based on a comparison of each dose level to the solvent control.

See page 43 for Legend to Cell Proliferation Kinetics.

Table 11a - *In Vitro* Sister Chromatid Exchange in CHO Cells

Compound	Dose (μg/ml)	S-9 (±)	No. Metaphases Scored	Range of SCE/Met[1]	Total Number of SCE's	Total Number of Chromosomes	SCE/ Chromosome	SCE/Met. S.D.
Untreated	0	-	50	5-26	700	991	0.71	14.000 ± 4.764
DMSO	1%	-	50	3-24	689	995	0.69	13.780 ± 4.171
MeNENA	50	-	50	8-20	685	995	0.69	13.700 ± 3.125
MeNENA	500	-	50	5-26	721	998	0.72	14.420 ± 5.167
MeNENA	1000	-	50	9-23	745	993	0.75	14.900 ± 3.448
MeNENA	2500	-	50	5-23	716	982	0.73	14.320 ± 4.727
MeNENA	5000	-	50	7-31	788	998	0.79	15.760 ± 5.575*
EMS	124	-	50	20-55	1712	995	1.72	34.240 ± 8.277**
Untreated	0	+	50	7-31	730	996	0.73	14.600 ± 4.806
DMSO	1%	+	50	8-34	806	997	0.81	16.120 ± 4.547
MeNENA	50	+	50	30-75	2418	998	2.42	48.360 ± 11.480**
MeNENA	100	+	50	39-91	3155	1002	3.15	63.100 ± 12.477**
MeNENA	500	+	50	53-107	4016	1001	4.01	80.320 ± 11.089**
MeNENA	1000	+	50	49-125	3834	994	3.86	76.680 ± 16.669**
MeNENA	2500	+	50	55-124	4099	989	4.14	81.980 ± 15.837**
DEN	100	+	50	11-48	1289	993	1.30	25.780 ± 8.200**

[1]Met = Metaphases
*,** Denotes a statistically significanct increase at $p \leq 0.05$, $p \leq 0.01$, respectively.

Table 11b - Cell Proliferation Kinetics Analysis

Compound	Dose (µg/ml)	S-9 (±)	Total No. Metaphases Scored	No. of Mitotic Divisions M1	M1+	M2	M2+	M3	MCC	APT (hrs.)	% APT Increase[1]
Untreated	0	-	200	4	7	124	60	5	2.14	13.55	-
DMSO	1%	-	200	3	3	151	42	1	2.09	13.88	-
MeNENA	50	-	200	7	6	142	40	5	2.08	13.94	0.43
MeNENA	500	-	200	6	6	150	38	0	2.05	14.15	1.95
MeNENA	1000	-	200	2	0	162	35	1	2.08	13.94	0.43
MeNENA	2500	-	200	2	7	127	62	2	2.14	13.55	-
MeNENA	5000	-	200	1	3	133	59	4	2.16	13.43	-
EMS	124	-	200	4	5	122	68	1	2.14	13.55	-
Untreated	0	+	200	2	7	122	69	0	2.15	13.48	-
DMSO	1%	+	200	7	9	121	61	2	2.11	13.74	-
MeNENA	50	+	200	6	11	152	31	0	2.02	14.36	4.51
MeNENA	100	+	200	5	7	177	11	0	1.99	14.57	6.04
MeNENA	500	+	200	9	24	164	3	0	1.90	15.26	11.06
MeNENA	1000	+	200	5	29	164	2	0	1.91	15.18	10.48
MeNENA	2500	+	200	16	37	145	2	0	1.83	15.85	15.36
DEN	100	+	200	6	12	157	25	0	2.00	14.50	5.53

Time in BrdUrd: 29 hours.
1 - % APT increase is based on a comparison of each dose level to the solvent control.
See page 43 for Legend to Cell Proliferation Kinetics.

Table 12a - In Vitro Sister Chromatid Exchange in CHO Cells

Compound	Dose (μg/ml)	S-9 (±)	No. Metaphases Scored	Range of SCE/Met[1]	Total Number of SCE's	Total Number of Chromosomes	SCE/ Chromosome	SCE/Met. S.D.
Untreated	0	-	50	8-25	764	1001	0.76	15.280 ± 3.839
DMSO	1%	-	50	6-27	759	995	0.76	15.180 ± 4.685
EtNENA	50	-	50	7-26	810	995	0.81	16.200 ± 4.932
EtNENA	250	-	50	9-29	914	997	0.92	18.280 ± 4.928**
EtNENA	500	-	50	9-36	1055	999	1.06	21.100 ± 6.004**
EtNENA	2000	-	50	15-68	1705	1000	1.71	34.100 ± 12.261**
EtNENA	5000	-	50	23-105	2735	994	2.75	54.700 ± 19.925**
EMS	124	-	50	14-73	1933	1000	1.93	38.660 ± 11.070**
Untreated	0	+	50	7-28	830	998	0.83	16.600 ± 4.986
DMSO	1%	+	50	6-31	813	996	0.82	16.260 ± 4.763
EtNENA	50	+	50	38-98	3030	1006	3.01	60.600 ± 11.925**
EtNENA	250	+	50	63-121	4498	997	4.51	89.960 ± 14.977**
EtNENA	500	+	50	61-132	4496	998	4.51	89.920 ± 16.199**
EtNENA	2000	+	50	63-110	4518	992	4.55	90.360 ± 12.008**
EtNENA	4000	+	50	75-156	5077	999	5.08	101.540 ± 16.314**
DEN	100	+	50	20-46	1535	999	1.54	30.700 ± 6.707**

[1]Met = Metaphases
*, **Denotes a statistically significanct increase at $p \leq 0.05$, $p \leq 0.01$, respectively.

Table 12b - Cell Proliferation Kinetics Analysis

Compound	Dose (μg/ml)	S-9 (±)	Total No. Metaphases Scored	No. of Mitotic Divisions M1	M1+	M2	M2+	M3	MCC	APT (hrs.)	% APT Increase[1]
Untreated	0	-	200	0	4	169	24	3	2.07	14.01	-
DMSO	1%	-	200	0	2	172	24	2	2.07	14.01	-
EtNENA	50	-	200	3	2	181	140	2.	0 2	14.36	2.50
EtNENA	250	-	200	0	1	182	16	1	2.04	14.22	1.50
EtNENA	500	-	200	2	5	174	18	1	2.03	14.29	2.00
EtNENA	2000	-	200	6	5	175	14	0	1.99	14.57	4.00
EtNENA	5000	-	200	6	8	152	32	2	2.04	14.22	1.50
EMS	124	-	200	2	3	158	37	0	2.08	13.94	-
Untreated	0	+	200	3	5	170	22	0	2.03	14.29	6.96
DMSO	1%	+	200	0	2	129	67	2	2.17	13.36	-
EtNENA	50	+	200	7	32	150	11	0	1.91	15.18	13.62
EtNENA	250	+	200	11	29	153	6	1	1.89	15.34	14.82
EtNENA	500	+	200	15	58	123	4	0	1.79	16.20	21.26
EtNENA	2000	+	200	42	94	64	0	0	1.56	18.59	39.15
EtNENA	4000	+	200	26	133	41	0	0	1.54	18.83	40.94
DEN	100	+	200	1	5	166	27	1	2.06	14.08	5.39

Time in BrdUrd: 29 hours.
1 - % APT increase is based on a comparison of each dose level to the solvent control.
See page 43 for Legend to Cell Proliferation Kinetics.

Table 13a - In Vitro Sister Chromatid Exchange in CHO Cells

Compound	Dose (μg/ml)	S-9 (±)	No. Metaphases Scored	Range of SCE/Met[1]	Total Number of SCE's	Total Number of Chromosomes	SCE/ Chromosome	SCE/Met. S.D.
Untreated	0	-	50	8-20	654	994	0.66	13.080 ± 3.036
DMSO	1%	-	50	4-25	675	998	0.68	13.500 ± 4.657
BuNENA	10	-	50	6-24	749	1002	0.75	14.980 ± 4.013*
BuNENA	50	-	50	6-23	690	989	0.70	13.800 ± 4.562
BuNENA	150	-	50	6-23	792	997	0.79	15.840 ± 4.068**
BuNENA	300	-	50	4-32	858	996	0.86	17.160 ± 5.523**
BuNENA	500	-	50	5-34	936	991	0.94	18.720 ± 5.621**
EMS	124	-	50	14-54	1658	1001	1.66	33.160 ± 9.686**
Untreated	0	+	50	9-29	861	999	0.86	17.220 ± 4.052
DMSO	1%	+	50	7-41	795	993	0.80	15.900 ± 5.665
BuNENA	10	+	50	10-54	1407	1005	1.40	28.140 ± 10.820**
BuNENA	50	+	50	22-73	2069	992	2.09	41.380 ± 10.740**
BuNENA	150	+	50	39-108	3277	998	3.28	65.540 ± 15.237**
BuNENA	300	+	50	43-109	3992	993	4.02	79.840 ± 14.313**
BuNENA	500	+	50	52-120	3821	990	3.86	76.420 ± 14.400**
DEN	100	+	50	12-64	1545	1000	1.55	30.900 ± 9.076**

[1]Met = Metaphases
*,**Denotes a statistically significanct increase at $p \leq 0.05$, $p \leq 0.01$, respectively.

Table 13b - Cell Proliferation Kinetics Analysis

Compound	Dose (μg/ml)	S-9 (±)	Total No. Metaphases Scored	No. of Mitotic Divisions M1	M1+	M2	M2+	M3	MCC	APT (hrs.)	% APT Increase[1]
Untreated	0	-	200	1	0	157	41	1	2.10	13.81	0.95
DMSO	1%	-	200	1	2	147	47	3	2.12	13.68	-
BuNENA	10	-	200	6	12	151	31	0	2.02	14.36	4.97
BuNENA	50	-	200	16	7	136	35	6	2.02	14.36	4.97
BuNENA	150	-	200	18	8	148	26	0	1.96	14.80	8.91
BuNENA	300	-	200	1	2	176	19	2	2.05	14.15	3.44
BuNENA	500	-	200	16	8	156	18	2	1.96	14.80	8.19
EMS	124	-	200	6	2	154	36	2	2.07	14.01	2.41
Untreated	0	+	200	0	0	149	51	0	2.13	13.62	-
DMSO	1%	+	200	0	1	160	39	0	2.10	1 3.81	-
BuNENA	10	+	200	7	5	159	28	1	2.03	14.29	3.48
BuNENA	50	+	200	4	10	173	13	0	1.99	14.57	5.50
BuNENA	150	+	200	6	20	169	5	0	1.93	15.03	8.83
BuNENA	300	+	200	9	18	172	1	0	1.91	15.18	9.92
BuNENA	500	+	200	24	24	152	0	0	1.82	15.93	15.35
DEN	100	+	200	4	4	157	34	1	2.06	14.08	1.96

Time in BrdUrd: 29 hours.
1 - % APT increase is based on a comparison of each dose level to the solvent control.
See page 43 for Legend to Cell Proliferation Kinetics.

Table 14a - In Vitro Sister Chromatid Exchange in CHO Cells

Compound	Dose (μg/ml)	S-9 (±)	No. Metaphases Scored	Range of SCE/Met[1]	Total Number of SCE's	Total Number of Chromosomes	SCE/ Chromosome	SCE/Met. S.D.
Untreated	0	-	50	6-22	663	999	0.66	13.260 ± 3.741
DMSO-1	1%	-	50	6-22	697	995	0.70	13.940 ± 3.925
DMSO-2	1%	-	50	5-27	670	995	0.67	13.400 ± 4.445
BDNPA/F +DPA	5	-	50	5-25	708	996	0.71	14.160 ± 4.325
BDNPA/F +DPA	25	-	50	8-21	750	996	0.75	15.000 ± 3.625
BDNPA/F +DPA	50	-	50	6-25	849	996	0.85	16.980 ± 4.172**
BDNPA/F +DPA	75[2]	-	50	9-28	860	993	0.87	17.200 ± 4.785**
BDNPA/F +DPA	100[2]	-	50	10-39	1036	986	1.05	20.720 ± 5.194**
EMS	124	-	50	18-69	1661	997	1.67	33.220 ± 9.466**
Untreated	0	+	50	5-30	759	997	0.76	15.180 ± 5.130
DMSO-1	1%	+	50	7-32	790	993	0.80	15.800 ± 5.299
DMSO-2	1%	+	50	6-23	613	997	0.61	12.260 ± 3.859
BDNPA/F +DPA	5	+	50	10-54	912	998	0.91	18.240 ± 7.358*
BDNPA/F +DPA	25	+	50	7-48	1053	993	1.06	21.060 ± 8.143**
BDNPA/F +DPA	50	+	50	4-45	861	988	0.87	17.220 ± 6.852
BDNPA/F +DPA	75[2]	+	50	7-40	831	993	0.84	16.620 ± 5.848
BDNPA/F +DPA	100[2]	+	50	9-28	861	995	0.87	17.220 ± 4.888**
DEN	100	+	50	14-45	1283	992	1.29	25.660 ± 7.697**

[1]Met = Metaphases
[2]Compared to DMSO-2 for statistical analysis
*,**Denotes a statistically significanct increase at $p \leq 0.05$, $p \leq 0.01$, respectively.

Table 14b - Cell Proliferation Kinetics Analysis

Compound	Dose (µg/ml)	S-9 (±)	Total No. Metaphases Scored	Nc. of Mitotic Divisions M1	M1+	M2	M2+	M3	MCC	APT (hrs.)	% APT Increase[1]
Untreated	0	-	200	5	0	165	30	0	2.05	14.15	0.50[a]
DMSO-1	1%	-	200	2	4	164	29	1	2.06	14.08	-
DMSO-2	1%	-	200	15	11	165	9	0	1.92	15.10	7.24[a]
BDNPA/F +DPA	5	-	200	14	8	163	15	0	1.95	14.87	5.61[a]
BDNPA/F +DPA	25	-	200	13	12	157	18	0	1.95	14.87	5.61[a]
BDNPA/F +DPA	50	-	200	41	33	123	3	0	1.72	16.86	19.74[a]
BDNPA/F +DPA	75	-	200	100	60	40	0	0	1.65	17.58	24.86[a]
BDNPA/F +DPA	100	-	200	116	53	31	0	0	1.29	22.48	48.87[b]
EMS	124	-	200	10	9	151	29	1	2.01	14.43	2.49[a]
Untreated	0	+	200	3	3	131	61	2	2.14	13.55	-
DMSO-1	1%	+	200	7	3	145	45	0	2.07	14.01	-
DMSO-2	1%	+	200	20	23	150	7	0	1.86	15.59	11.28[a]
BDNPA/F +DPA	5	+	200	3	4	176	17	0	2.02	14.36	2.50[a]
BDNPA/F +DPA	25	+	200	6	9	162	23	0	2.01	14.43	3.00[a]
BDNPA/F +DPA	50	+	200	20	13	160	7	0	1.89	15.34	9.49[a]
BDNPA/F +DPA	75	+	200	122	14	63	1	0	1.36	21.32	52.18[a]
BDNPA/F +DPA	100	+	200	57	43	99	1	0	1.61	18.01	15.52[b]
DEN	100	+	200	1	8	138	53	0	2.11	13.74	-

Time in BrdUrd: 29 hours.
1 - % APT increase is based on a comparison of each dose level to the solvent control.
See page 43 for Legend to Cell Proliferation Kinetics. All dose levels and controls were compared to DMSO-1 except for 100 ug/ml (with and without S-9) which was compared to DMSO-2 along with the untreated cultures.
[a]Increase compared to DMSO-1.
[b]Increase compared to DMSO-2.

Table 15a - In Vitro Sister Chromatid Exchange in CHO Cells

Compound	Dose (μg/ml)	S-9 (±)	No. Metaphases Scored	Range of SCE/Met[1]	Total Number of SCE's	Total Number of Chromosomes	SCE/ Chromosome	SCE/Met. S.D.
Untreated	0	-	50	10-26	859	1002	0.86	17.180 ± 4.434
DMSO	1%	-	50	8-28	863	994	0.87	17.260 ± 4.840
BDNPA/F -DPA	5	-	50	5-27	745	997	0.75	14.900 ± 4.962
BDNPA/F -DPA	10	-	50	6-33	814	992	0.82	16.280 ± 5.707
BDNPA/F -DPA	25	-	50	6-38	887	994	0.89	17.740 ± 6.452
BDNPA/F -DPA	40	-	50	7-30	881	995	0.89	17.620 ± 5.241
BDNPA/F -DPA	50	-	50	7-24	801	990	0.81	16.020 ± 3.750
EMS	124	-	50	17-53	1588	998	1.59	31.760 ± 7.862**
Untreated	0	+	50	5-23	704	996	0.71	14.080 ± 4.149
DMSO	1%	+	50	7-25	759	996	0.76	15.180 ± 4.232
BDNPA/F -DPA	5	+	50	7-33	784	996	0.79	15.680 ± 5.065
BDNPA/F -DPA	10	+	50	6-26	770	993	0.78	15.400 ± 5.272
BDNPA/F -DPA	25	+	50	5-35	796	1000	0.80	15.920 ± 5.439
BDNPA/F -DPA	40	+	50	10-47	1038	998	1.04	20.760 ± 6.511**
BDNPA/F -DPA	50	+	50	11-48	1000	991	1.01	20.000 ± 6.878**
DEN	100	+	50	12-54	1546	998	1.55	30.920 ± 8.121**

[1]Met = Metaphases
*, ** Denotes a statistically significant increase at $p \leq 0.05$, $p \leq 0.01$, respectively.

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Table 15b - Cell Proliferation Kinetics Analysis

Compound	Dose (µg/ml)	S-9 (±)	Total No. Metaphases Scored	No. of Mitotic Divisions M1	M1+	M2	M2+	M3	MCC	APT (hrs.)	% APT Increase[1]
Untreated	0	-	200	21	11	159	9	0	1.89	15.34	-
DMSO	1%	-	200	27	18	153	2	0	1.83	15.85	-
BDNPA/F -DPA	5	-	200	13	12	175	0	0	1.91	15.18	-
BDNPA/F -DPA	10	-	200	30	25	142	3	0	1.80	16.11	1.64
BDNPA/F -DPA	25	-	200	15	23	153	9	0	1.89	15.34	-
BDNPA/F -DPA	40	-	200	23	18	154	5	0	1.85	15.68	-
BDNPA/F -DPA	50	-	200	48	52	96	4	0	1.64	17.68	11.55
EMS	100	-	200	26	22	146	6	0	1.83	15.85	-
Untreated	0	+	200	29	38	127	6	0	1.78	16.29	15.70
DMSO	1%	+	200	1	7	161	31	0	2.06	14.08	-
BDNPA/F -DPA	5	+	200	20	40	125	15	0	1.84	15.76	11.93
BDNPA/F -DPA	10	+	200	15	29	156	0	0	1.85	15.68	11.36
BDNPA/F -DPA	25	+	200	18	30	150	2	0	1.84	15.76	11.93
BDNPA/F -DPA	40	+	200	50	45	101	4	0	1.65	17.58	24.86
BDNPA/F -DPA	50	+	200	63	52	85	0	0	1.56	18.59	32.03
DEN	100	+	200	35	82	75	8	0	1.64	17.68	25.57

Time in BrdUrd: 29 hours.

1 - % APT increase is based on a comparison of each dose level to the solvent control.

See page 43 for Legend to Cell Proliferation Kinetics.

Table 16. Summary of SCE Results

Test Article	±S-9	Dose (μg/ml)	Statistically Significant	Dose Dependent	Fold Increase
MeNENA	-	50-5000	yes	yes	no
	+	50-2500	yes	yes	5.1-fold
EtNENA	-	50-5000	yes	yes	3.6-fold
	+	50-4000	yes	yes	6.3-fold
BuNENA	-	10-500	yes	yes	no
	+	10-500	yes	yes	5.0-fold
BDNPA/F+DPA	-	5-100	yes	yes	no
	+	5-100	yes	no	no
BDNAP/F-DPA	-	5-50	no	no	no
	+	5-50	yes	no	no

Induction of SCEs without S-9

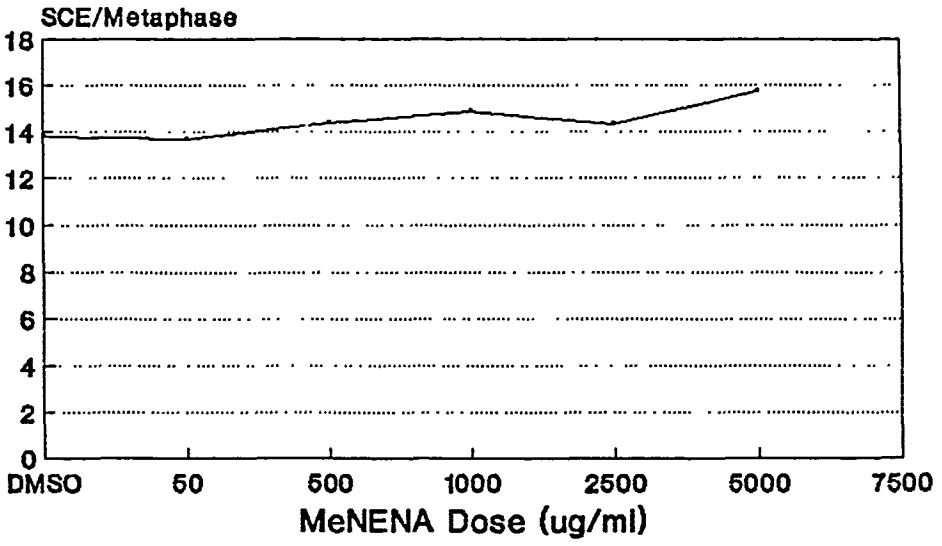

Figure 1A

Induction of SCEs with S-9

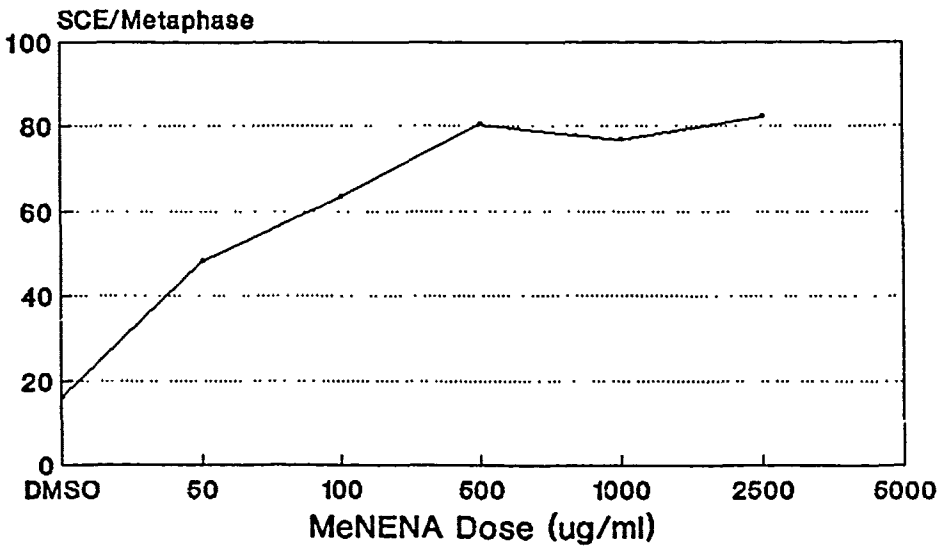

Figure 1B

Induction of SCEs without S-9

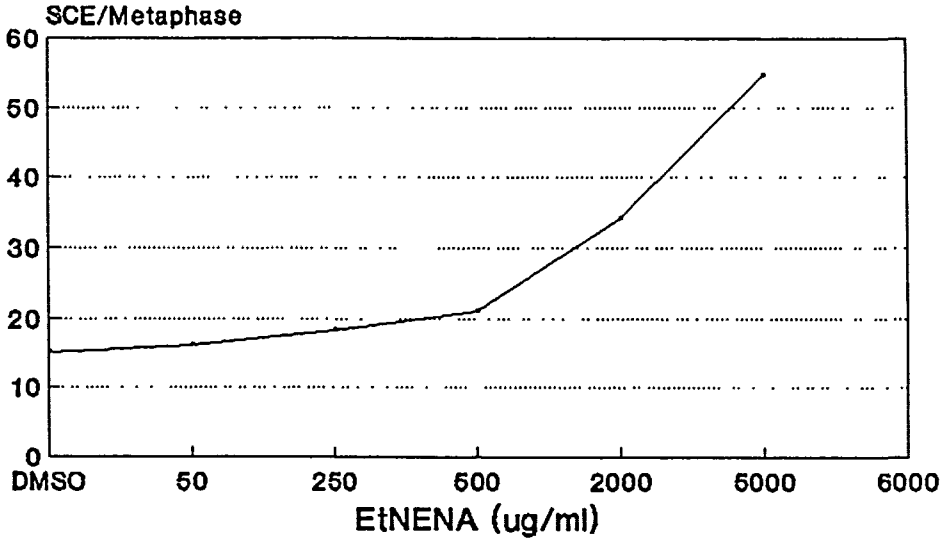

Figure 2A

Induction of SCEs with S-9

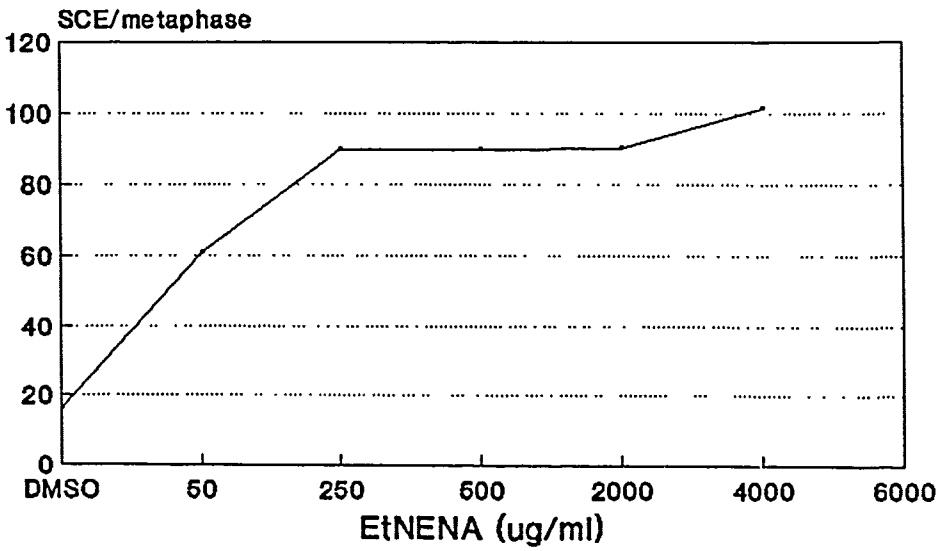

Figure 2B

Induction of SCEs without S-9

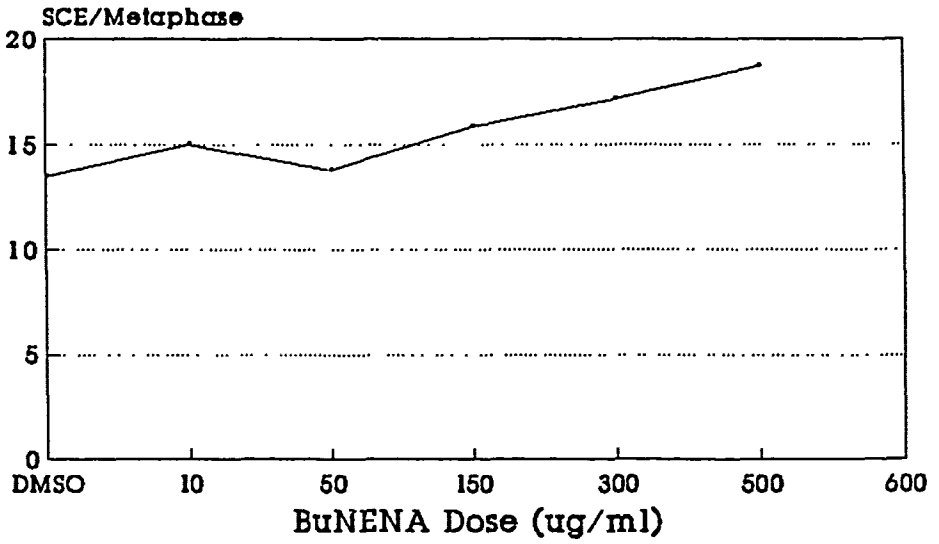

Figure 3A

Induction of SCEs with S-9

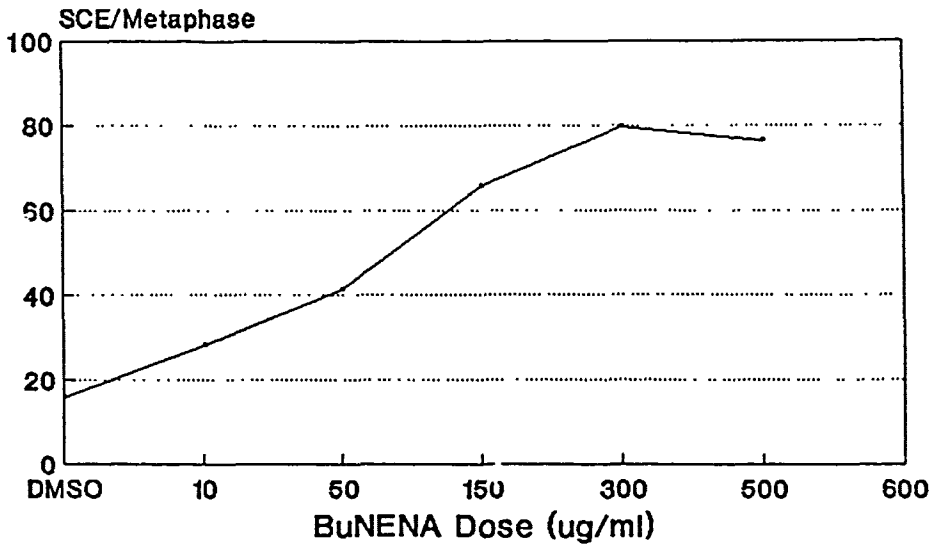

Figure 3B

Induction of SCEs without S-9

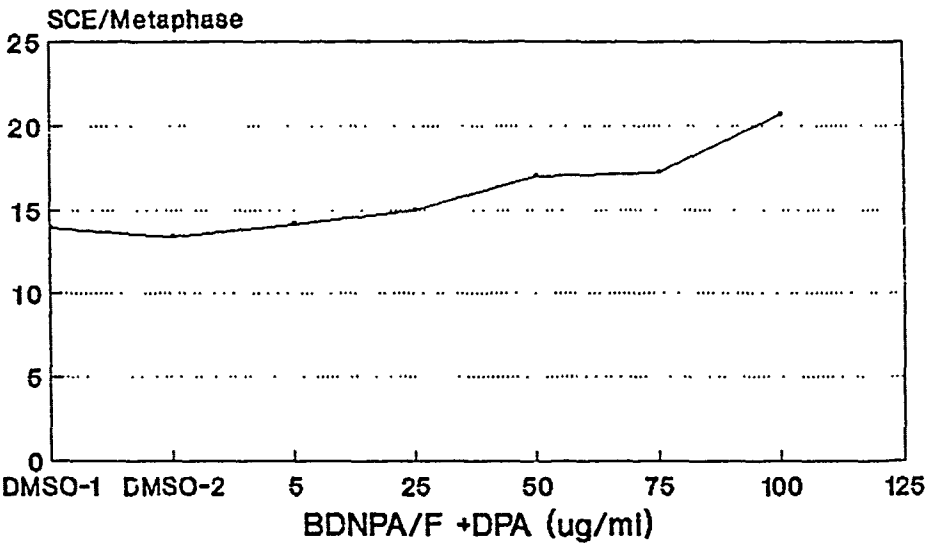

Figure 4A

Induction of SCEs with S-9

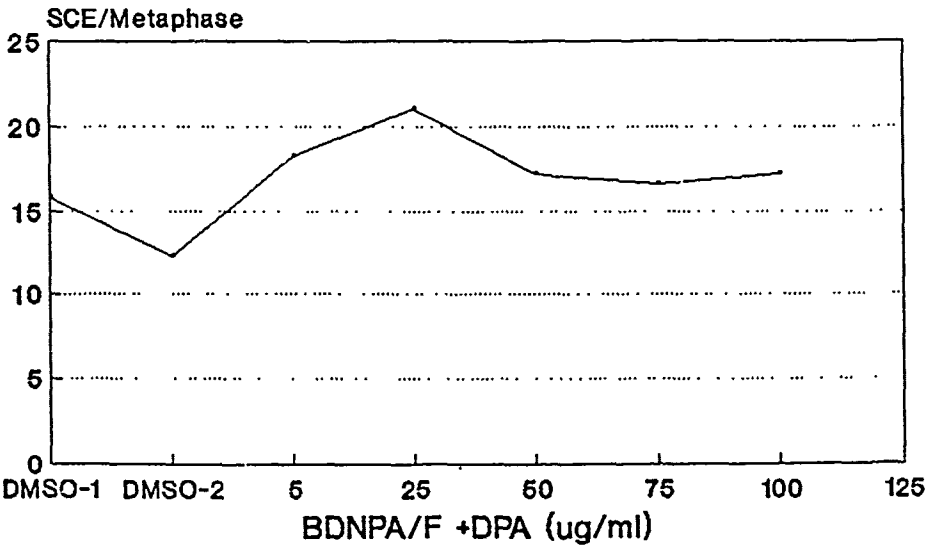

Figure 4B

Induction of SCEs without S-9

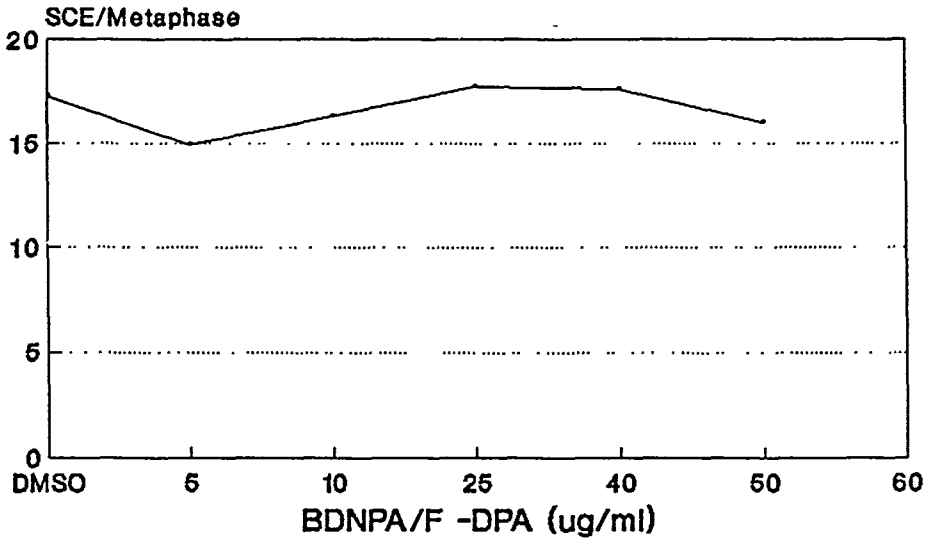

Figure 5A

Induction of SCEs with S-9

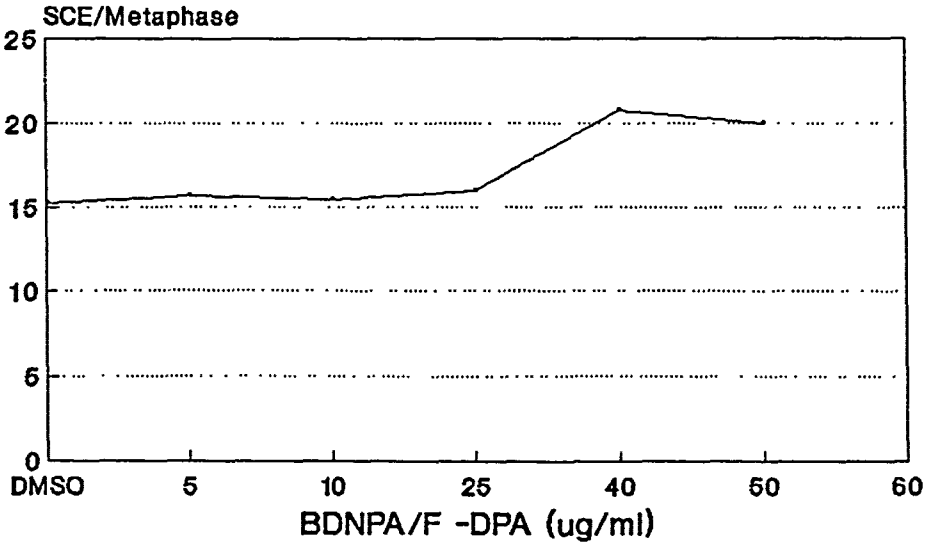

Figure 5B

Legend to Cell Proliferation Kinetics

M1 = Cells that have replicated one complete cell cycle in the presence of BrdUrd.

M1+ = Cells that have replicated between one and two cell cycles in the presence of BrdUrd.

M2 = Cells that have replicated two complete cell cycles in the presence of BrdUrd.

$M2^+$ = Cells that have replicated between two and three cell cycles in the presence of BrdUrd.

M3 = Ratio of approximately 1/4 dark staining chromatids 3/4 light staining chromatids.

$$\text{MCC} = \text{Mean Cell Cycle} = \frac{1M_1 + 1.5M_1^+ + 2M_2 + 2.5M_2^+ + 3M_3}{\text{\# Metaphases Scored}}$$

$$\text{APT} = \text{Average Proliferation Time} = \frac{\text{time in BrdUrd (hours)}}{\text{MCC}}$$

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COMPLIANCE STATEMENT

Except that analytical analyses of dosing solutions were not performed to verify the accuracy or stability of the test article dosing solutions, this study was conducted in compliance with the Principles of Good Laboratory Practices (GLP) as promulgated by the following regulatory agencies:

U.S. Food and Drug Administration, as stated in the Federal Register, 21 CFR Part 58, Friday, September 4, 1987.

U.S. Environmental Protection Agency as stated in the Federal Register, 21 CFR Part 58.

U.S. Environmental Protection Agency as stated in the Federal Register, 40 CFR Parts 160 and 792.

Organization for Economic Co-operation and Development Guidelines for Testing Chemicals (OECD), ISBN 92-64 12221-4.

Study Nos.: PH 301-US-001-91
PH 301-US-002-91
PH 301-US-003-91
PH 301-US-004-91
PH 301-US-005-91

"To the best of my knowledge, this study was conducted in accordance with applicable Good Laboratory Practice regulations; there were no deviations from these regulations that impacted on study conclusions."

Juan Sebastian
Study Director

May 27, 1992
Date

PHARMAKON RESEARCH INTERNATIONAL, INC.
Waverly, PA 18471

QUALITY ASSURANCE UNIT STATEMENT

STUDY NUMBER: PH 319-US-001-91
PH 319-US-002-91
PH 319-US-003-91
PH 319-US-004-91
PH 319-US-005-91

STUDY DIRECTOR: Juan R. SanSebastian, Ph.D.

STUDY TITLE: Evaluation of Five Unicharge Propellants in the In Vitro Sister Chromatid Exchange Assay in Chinese Hamster Ovary Cells

The following study inspections have been performed by the QAU and the results have been reported to the study director and management on the date(s) indicated.

The following inspections were performed:

Phase	Date(s)
Treatment	October 10, 1991
Reporting	January 22, 1992

Date(s) QAU Report Issued To:

STUDY DIRECTOR: January 22, 1992

MANAGEMENT: January 22, 1992

Leslie J. Pinnell, M.S.
Manager, Quality Assurance

May 27, 1992
Date