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Genotoxicity of the cancer chemopreventive drug candidates CP-31398, SHetA2, and phospho-ibuprofen

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Abstract

The genotoxic activities of three cancer chemopreventive drug candidates, CP-31398 (a cell permeable styrylquinazoline p53 modulator, SHetA2 (a flexible heteroarotinoid), and phospho-ibuprofen (PI, a derivative of ibuprofen) were tested. None of the compounds were mutagenic in the *Salmonella*/*Escherichia coli*/microsome plate incorporation test. CP-31398 and SHetA2 did not induce chromosomal aberrations (CA) in Chinese hamster ovary (CHO) cells, either in the presence or absence of rat hepatic S9 (S9). PI induced CA in CHO cells, but only in the presence of S9. PI, its parent compound ibuprofen, and its moiety diethoxyphosphoryloxybutyl alcohol (DEPBA) were tested for CA and micronuclei (MN) in CHO cells in the presence of S9. PI induced CA as well as MN, both kinetochore-positive (Kin+) and -negative (Kin−), in the presence of S9 at ≤100 μg/ml. Ibuprofen was negative for CA, positive for MN with Kin+ at 250 μg/ml, and positive for MN with Kin− at 125 and 250 μg/ml. DEPBA induced neither CA nor MN at ≤5000 μg/ml. The induction of chromosomal damage in PI-treated CHO cells in the presence of S9 may be due to its metabolites. None of the compounds were genotoxic, in the presence or absence of S9, in the GADD45α-GFP Human GreenScreen assay and none induced MN in mouse bone marrow erythrocytes.

Keywords

Chemopreventive agents; genotoxicity; mutagenticity; chromosomal aberrations; micronuclei

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

1. Introduction

Many cancer chemotherapeutic drugs are DNA-damaging agents, causing side effects and increasing the risk of secondary cancers. Recently, several small molecules, including CP-31398, a p53 modulator, SHetA2, a flexible heteroarotinoid, and phospho-ibuprofen (PI), a novel derivative of ibuprofen, have been evaluated by the National Cancer Institute (USA; NCI) Division of Cancer Prevention as candidate chemopreventive and chemotherapeutic agents. The Rapid Access to Preventive Intervention Development Program (RAPID) or PREVENT program, with the help of the Chemopreventative Agent Development Research Group (CADRG) in the Division of Cancer Prevention (DCP) of the NCI supports the identification and development of chemopreventive agents, from synthesis to initial testing in humans, including the preclinical toxicology testing required for Investigational New Drug (IND) application submission to the U.S. Food and Drug Administration (FDA) [1]. CP-31398 has been shown to trigger p53-dependent cell death pathways in cancer cell lines [2; 3; 4], and to suppress tumors in mice [5; 6]. SHetA2 is known to induce growth inhibition and apoptosis in cell lines and in animal models [7]. Novel phospho-nonsteroidal anti-inflammatory drugs (NSAIDS), including phospho-ibuprofen, has been a significantly effective chemopreventive agent against several cancers [8; 9]. Based on these characteristics, CP-31398, SHetA2, and PI were selected by the CADRG group for preclinical toxicology testing as possible chemopreventive drugs.

Genetic damage, including chromosomal aberrations, plays a major role in neoplastic development [10; 11]. A relevant biomarker for cancer risk in humans is induction of chromosomal aberrations in peripheral blood lymphocytes [12; 13]. Identifying possible genotoxic effects of chemopreventive agents is important for the risk/benefit assessment of their potential use in humans. This study evaluates these compounds for genotoxic potential, in the *in vitro Salmonella-E. coli* mutagenicity assay, the Chinese hamster ovary cell chromosome aberration assay (CHO-CA), and the *in vivo* mouse bone marrow micronucleus assay. The International Conference on Harmonization (ICH) [14], FDA, and other regulatory agencies [15] recommend these assays. The PI moieties ibuprofen and diethoxyphosphoryloxybutyl alcohol (DEPBA) were further tested in the GADD45α-GFP Human GreenScreen assay.

2. Materials and Methods

2.1. Chemicals

CP-31398, N-{2-[2-(4-methoxy-phenyl)-vinyl]-quinazoline-4-yl}-N,N-dimethyl-propane-1,3-diamine HCl was manufactured at INDOFINE Chemical Co. Inc. (Hillsborough, NJ), and the other three test articles, SHetA2 (1-(4-nitrophenyl)-3-(2,2,4,4-tetramethyl-3,4-dihydro-2*H*-thiochromen-6-yl) thiourea), phospho-ibuprofen (PI; 2-(4-isobutylphenyl)-propionic acid 4-(diethoxyphosphoryloxy)-butyl ester), and diethoxyphosphoryloxy butyl alcohol (DEPBA) were manufactured at Onyx Scientific Ltd. (Sunderland, UK). CP-31398, SHetA2, PI, and DEPBA were obtained from Fisher Bioservices (Germantown, MD). Ibuprofen, sodium azide (CAS No. 26628-22-8), 2-aminoanthracene (CAS No. 613-13-8), methyl methanesulfonate (MMS, CAS No. 66-27-3), cyclophosphamide (CAS No.6055-19-2), and urethane (CAS No. 51-79-6) were obtained from Sigma Chemical Co. (St. Louis, MO); 9-aminoacridine hydrochloride (CAS No. 52417-22-8), 2-nitrofluorene (CAS No. 607-57-8), 4-nitroquinoline N-oxide (CAS No. 56-57-5), and 20-methylcholanthrene (MCA, CAS No. 766-40-5) were obtained from Aldrich Chemical Co (Milwaukee, WI); and rat S9 was obtained from Molecular Toxicology, Inc., (Boone, NC).

2.2. Solvent/Vehicle controls

Sterile water (Baxer Healthcare, Deerfield, IL) was the solvent for CP-31398. Dimethyl sulfoxide (DMSO; Mallinckrodt, Phillipsburg, NJ) was the solvent for SHetA2, PI, ibuprofen, and DEPBA, for *in vitro* studies. For the *in vivo* mouse bone marrow micronucleus assay, methyl cellulose, 400 CPS USP (Sigma-Aldrich Inc., St. Louis, MO) was the solvent for CP-31398 and SHetA2, and PEG 400 (Spectrum Chemicals, New Brunswick, NJ) for PI.

The *Salmonella-Escherichia coli*/microsome plate incorporation test (Ames test), CHO chromosomal aberration assays, and *in vivo* mouse bone marrow micronucleus assays were conducted in compliance with Good Laboratory Practice (GLP) and the International Conference on Harmonization (ICH) Guidelines. PI, ibuprofen, and DEPBA, in the presence of S9, were further tested in the *in vitro* micronucleus assay with antikinetochore antibody labeling. In addition, the GADD45α-GFP GreenScreen Human Cell based genotoxicity assay (GreenScreen HC assay) was conducted with PI, ibuprofen, and DEPBA.

2.3 Ames test

Salmonella typhimurium LT2 strains (TA1535, TA1537, TA98, and TA100) were obtained from Dr. Bruce Ames (University of California, Berkeley), and *E. coli* strain WP2 (*uvr*A) was obtained from the National Collection of Industrial and Marine Bacteria (Aberdeen, Scotland). Strains were kept frozen at −80°C, in nutrient broth supplemented with 10% sterile glycerol. Experiments with *Salmonella* and *E. coli* strains were performed as described previously [16; 17]. The standard plate incorporation procedure was used [18]. Briefly, for each test article, a dose range finding and two mutagenicity experiments were conducted in the presence or absence of rat S9, up to a maximum concentration of 5 mg/plate, using triplicate plates per concentration. The positive controls in the absence of S9 were sodium azide (TA1535 and TA100), 9-aminoacridine hydrochloride (TA1537), 2-nitrofluorene (TA98), and 4-nitroquinoline N-oxide [WP2 (*uvr*A)]. In the presence of S9, for all strains, the positive control was 2-aminoanthracene. Statistical analysis was performed using Levene's test, the one-tailed Dunnett's *t*-test, and evaluation of dose-relatedness by regression analysis, using a *t*-statistic to test the significance of the regression. The statistical analyses were performed using the SAS analysis system. Results were considered positive if reproducible and statistically significant ($p<0.01$) increases in revertants at one or more concentrations, and dose-related increases in the numbers of revertants, were observed.

2.4. In vitro chromosome aberration assay with Chinese hamster ovary cells (CHO)

CHO cells (ATCC CCL 61 CHO-K1, proline-requiring) were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in an atmosphere of 5% CO_2 at 37°C in F-12 medium with 10% fetal bovine serum (FBS), 2 mM GlutaMAX, and 1% penicillin-streptomycin solution to maintain exponential growth. Cells were grown in this medium during exposure to dose formulations without S9. F-12 medium with 2.5% FBS (containing GlutaMAX and penicillin-streptomycin in the above concentrations) was used to grow cells exposed to dose formulations with S9. For each test article, a dose range finding and two chromosome aberration experiments were conducted in the presence and absence of rat metabolic activation (S9), up to a maximum concentration of 5 mg/ml. Duplicate cultures were maintained for each concentration in the presence or absence of S9 and for each exposure condition. The exposure periods were 3 and 21 h without S9, and 3 h with S9. MMS was used in the absence of S9 and cyclophosphamide in the presence of S9 as positive controls. The standard procedure was used for harvesting cultures and analyzing metaphases [18; 19; 20]. 1000 cells for toxicity, and 200 cells for metaphases per concentration were scored in each experiment. The types of aberrations observed were chromatid and chromosome gaps, breaks, exchanges, deletions, acentric fragments and dicentrics;

chromatid and chromosome gaps were not included in total aberrations. In addition to these aberrations, polyploidy and endoreduplications were also observed. For statistical analysis, the number of cells with structural chromosome damage observed in the test article and the positive control treatment groups were compared with those for the concurrent solvent control group, using Fisher's Exact test (significance level of $p < 0.05$, one-tailed) [FISHEX.PL.XLT v. 1.0 macro run on MS Excel v. 5.0]. The Cochran-Armitage test ($p < 0.01$) was used to calculate the dose-related response. A test article was considered positive if there was a statistically significant increase ($p < 0.05$) in the frequency of cells with structural chromosomal damage at one or more concentrations, and this increase was dose related and reproducible. A test article was considered negative if the criteria for a positive response was not met.

2.5. In vitro micronucleus assay with CHO Cells with antikinetochore antibody labeling

CHO cultures were set up as mentioned in the chromosomal aberration assay. Ibuprofen, PI, DEPBA, solvent, and positive articles were added to cells in exposure medium and incubated in a 5% CO_2 environment at 37°C for 3 h in the presence of S9. Cells were then rinsed and allowed to incubate an additional 18 h in exposure medium with cytochalasin B. After incubation, cultures were harvested and cells were removed using trypsin, followed by the addition of fresh culture medium and the resulting cell suspension decanted into 15 ml centrifuge tubes. Cells were applied to slides using a cytocentrifuge, allowed to dry for 15 min before they were fixed by dipping into 100% methanol for 10 sec, and repeated once after drying. Slides were stored at 2°–8°C until antibody labeling.

The standard procedure was used for antibody labeling and for scoring micronuclei [21]. Briefly, slides were soaked in PBS with 0.1% tween 20 twice, for 2 min each time. After draining excess liquid, antikinetochore antibody solution (1:1 in PBS with 0.2% tween 20; 20 μl) was added to the surface of the slide and incubated in a humidified chamber at 37°C with a plastic coverslip for 1 h. The coverslip was removed and the slides were soaked two times for 2 min each in PBS with 0.1% tween 20. After draining excess liquid from the slides, FITC-conjugated goat anti-human IgG antibody solution (1:120 in PBS with 0.5% tween 20; 20 μl) was added to each slide. The slides were incubated in a humidified chamber at 37°C with a plastic coverslip for 1 h, and washed in the same manner as after the antikinetochore antibody incubation. While the slides were wet, 20 μl of DAPI (4′,6-diamidino-2-phenylindole with anti-fade; 0.5 μg/ml) was added, and a glass coverslip placed on top. The slides were refrigerated until coding and scoring. For analysis, a total of 1000 binucleated (BN) cells per culture were analyzed for proliferation index. At least 1000 cells per culture were scored for the presence of micronuclei and for the presence or absence of kinetochores in micronuclei. The number of cells with micronuclei observed in the test article, and positive control treatment groups were statistically compared with those of the concurrent solvent control group using Fisher's Exact test (significance level of $p < 0.05$, 1-tailed). Dose-related responses were calculated using the Cochran-Armitage test ($p < 0.01$).

2.6. In vivo mouse bone marrow micronucleus assay

Male and female Swiss-Webster mice were obtained from Charles River Laboratories (Kingston, NY; Portage, MI), and were approximately 7 weeks of age at the time of dosing. Animals were maintained in clear polycarbonate cages with hardwood chip bedding, and were provided Purina Rodent Chow and tap water *ad libitum*. All animal work was approved by SRI's Institutional Animal Care and Use Committee (IACUC) in full compliance with all regulations of the NIH Office of Laboratory Animal Welfare (OLAW). Animals were given a single oral administration of vehicle or test article since it is the route of human exposure. A dose range finding experiment with 3 mice/sex/treatment group with 48 h exposure and a micronucleus experiment with 5 mice/sex/treatment group with 24 and 48 h exposure were

performed using a highest dose level of 2 g/kg. Slides were prepared and stained using acridine orange [22; 23; 24]. Vehicle control and urethane positive control (300 mg/kg) groups were maintained simultaneously. For each animal, 200 cells were scored to determine the ratio of polychromatic erythrocytes (PCE) to red blood cells (RBC), and 2,000 PCE were scored for micronuclei (MN). Cochran-Armitage trend test and the normal test for equality of binomial proportions were used for statistical analysis. A test article was considered positive if there was a statistically significant increase ($p < 0.05$) in micronucleated PCE, the increase was dose-related, and the micronucleated PCE frequency was greater than the mean historical micronucleus frequency ± 2 standard deviations (SD). The test article was considered negative if the criteria for a positive response was not met.

2.7. GADD45α-GFP GreenScreen human cell assay (GreenScreen HC assay)

The GreenScreen HC assay is a high-specificity and high-sensitivity genotoxicity assay that can identify mutagens, clastogens, aneugens, and other genotoxic damages [25]. This assay uses the GFP (Green Fluorescent Protein) reporter to measure transcription of the GADD45α gene. GADD45α is a human protein involved in DNA damage and repair, apoptosis, and cell cycle control. Following exposure to genotoxic stress, the GADD45α gene is transcriptionally induced and GFP is produced [25]. Two human cell lines, GenM-C01 and GenM-T01 (Gentronix©, Manchester, UK), were used in the assay. The plasmids are stably maintained in TK6 cells by addition of 200 μg/ml hygromycin B to the cultures and incubated at 37°C with 5% CO_2. The test system without S9 was exposed to the test article via a microplate format described by Hastwell *et al.* [25]. The test system with S9 was exposed to the test article via a microplate format described by Jagger *et al.* [26]. Following the 3 h exposure, the microplate was washed twice with pre-warmed phosphate-buffered saline and the cells were resuspended in GreenScreen Recovery media. The microplate was covered with a breathable membrane and incubated in a humidified, 5% CO_2 incubator set at 37 °C for approximately 45 h.

For data collection in the absence of S9, the GFP-reporter fluorescence and cell culture absorbance data were collected from the microplates approximately 24 and 48 h after initiation of treatment. Absorbance data were collected using a spectrophotometer (Tecan-Safire) from the same plate at the 24 and 48 h time points. The data were entered into a spreadsheet template obtained from Gentronix (Manchester, UK). For collection in the presence of S9, the GFP-reporter fluorescence and cell culture absorbance data were collected from the microplates at approximately 48 h after initiation of treatment by means of flow (BD - FACS-Canto) cytometry. Green fluorescent protein was used for the measurement of fluorescence and propidium iodide was used for the measurement of cell population. Fluorescence data were derived from 10,000 events collected in the flow cytometry gate “NOT Debris AND NOT Dead Cells”. The data were entered into a spreadsheet template obtained from Gentronix (Manchester, UK). Data analysis was performed using the methods described by Hastwell *et al.* [25]. Using absorbance data normalized to untreated controls (=100% growth), as an indication of reduction in relative cell density, fluorescence data was divided by absorbance to give brightness, the measure of the average GFP induction per cell. Data for both strains, GenM-C01 and GenM-T01, are thus calculated and normalized to the untreated control (=1). They were then corrected for induced cellular auto-fluorescence and intrinsic test article fluorescence by subtracting the brightness values of GenM-C01 from GenM-T01.

In the absence of S9, the test article was considered positive for genotoxicity if the relative GFP induction ratio was greater than the 1.5 threshold (i.e., greater than 3 times the standard deviation of the background brightness [25]; and, in the presence of S9, if the relative GFP induction ratio was greater than the 1.3 threshold (i.e., a 30% increase of GFP expression) [26].

3. Results

The genotoxic potentials of CP-31398, SHetA2, and PI were evaluated using the Ames test, the chromosomal aberration assay with CHO cells, and the mouse bone marrow micronucleus assay. The PI parent compound ibuprofen and its moiety DEPBA were evaluated for chromosomal aberrations and *in vitro* micronucleus assay. The GreenScreen HC assay was performed to evaluate the transcription of the GADD45α gene induced by PI, ibuprofen, and DEPBA. An overview of the genotoxicity of the three compounds evaluated in this study is given in Table 1.

The results of the positive controls in the Ames test were acceptable for all experiments, as they elicited a response ≥ 5-fold increase over the mean value for the solvent. In the chromosomal aberration study, solvent control values were within the historical range (0 – 5.0% aberrations) and the positive controls (methyl methanesulfonate and cyclophosphamide) produced statistically significant increases ($p<0.05$) in the number of cells with structural chromosome aberrations. Vinblastine (the aneugenic positive control in the *in vitro* micronucleus experiment) produced statistically significant increases ($p<0.05$) in the number of micronucleated binucleate cells and 90.3 to 95% of these cells were kinetochore positive, indicating a predominantly aneugenic response. Cyclophosphamide (the clastogenic positive control), in the *in vitro* micronucleus experiment produced a statistically significant increase ($p<0.05$) in micronucleated cells and 70.8 to 92.7% of these cells were without kinetochores, indicating a predominantly clastogenic response. Urethane, a positive control for mouse bone marrow micronucleus studies induced micronuclei significantly (p<005).

3.1. CP-31398

The Ames test was performed in a dose range 39.1–2,500 μg/plate. Cytotoxicity was seen at doses ≥1,250 μg/plate in the presence and absence of rat metabolic activation (S9). The results are presented in Table 2. The first experiment for mutagenicity was conducted with all five tester strains, TA1535, TA1537, TA98, TA100, and *E. coli* strain WP2 (*uvr*A), with doses ranging from 78.1–2,500 μg/plate in the presence or absence of S9. No statistically significant increase in the number of revertant colonies was seen with any of the strains. The second experiment for mutagenicity was conducted in the presence or absence of S9 over a dose range 39.1–2,500 μg/plate. No statistically significant increase in the number of revertant colonies was seen for any of the strains, except for TA100 in the presence of S9 at 156.3 and 312.5 μg/plate. These increases were statistically significant ($p < 0.01$); however, they were determined not to be dose-dependent. Because the increase was less than 2-fold over solvent control values, within the historical spontaneous range for the strain, and not dose-dependent, they were not considered to be biologically significant.

In the chromosomal aberration assay, a cytotoxicity experiment was conducted at concentrations 1.95–5,000 μg/ml in the presence of S9 for 3 h exposure and for 3 and 21 h in the absence of S9. Cytotoxicity (<50% confluency or a >50% reduction in mitotic index) was observed at ≥78.1 μg/ml. Cells were tested at 0.625–40 μg/ml for 3 h exposure in the presence and absence of S9. Results are presented in Fig. 1. In the absence of S9, cells were analyzed at 2.5, 5, and 10 μg/ml for chromosomal aberrations, resulting in 3%, 1.5%, and 2%, respectively. There was no significant increase in cells with aberrations at any concentration compared with controls (1%). The absence of S9 was repeated with 21 hr exposure and no significant increases in cells with aberrations were observed at 1.25, 2.5, or 5 μg/ml, with 1%, 1.5%, 1%, respectively, and 1.5% in control. In the presence of S9, cells were analyzed for chromosomal aberrations at 10 (1%), 20 (1.5%), and 40 (2%) μg/ml, and no significant increase in chromosomal aberrations or dose-related effect, compared with control (2%), was observed. These results were reproducible in the second experiment

(1.5%, 1.5%, and 3% vs 2.5% in controls) at the same concentrations. Positive control values for MMS 50 μg/ml and CP 12.5 μg/ml in the first experiment, and MMS 20 μg/ml and CP 12.5 μg/ml in the second experiment, were all significant, with 36%, 34%, 46%, and 38% cells with aberrations, respectively. In addition, no dose-related increase in polyploidy was observed in the presence or absence of S9 (Table 8).

In the mouse bone marrow micronucleus assay, a dose range-finding experiment was conducted at 50–2,000 mg/kg CP-31398. No suppression of polychromatic erythrocytes (PCE) among total red blood cells (RBC) in either sex at ≤1,000 mg/kg was observed. All male and female mice in the 2,000 mg/kg dose group were found dead. In the micronucleus experiment, male and female mice were exposed to a single administration of CP-31398 at dose levels of 250, 500, and 1000 mg/kg, sacrificed 24 or 48 hr later, and the bone marrow was then evaluated for cytotoxicity and micronucleus formation. Results are presented in Table 5. There was no significant suppression of PCE in either sex at any dose level. No statistically significant increase in the frequency of micronucleated PCE was seen at any dose level in either male or female mice at the 24 or 48 h time point.

3.2. SHetA2

In the Ames test, cytotoxicity was observed at ≥9.77 μg/plate for strain TA100 in the absence of S9 and ≥19.53 μg/plate in the presence of S9. In the first experiment, no statistically significant increases in the number of revertant colonies were observed from 0.31–19.53 μg/plate. The results are presented in Table 3. Cytotoxicity varied with the *Salmonella* strains under the various test conditions; however, no cytotoxicity was seen with the *E. coli* strain. Therefore, doses in the second experiment with the *Salmonella* strains were 0.31–9.77 μg/plate in the absence of S9, 1.22–78.13 μg/plate in the presence S9, and 2.44–156.3 μg/plate for the *E. coli* strain, with or without S9. No statistically significant increases in the number of revertant colonies were seen for any of the strains, except for TA98 in the presence of S9. The increase was dose-dependent; however, no colony counts were found to be significant. Because the increase was less than 2-fold over solvent control values and within the historical spontaneous range for the strain, it was not considered to be biologically significant.

In the chromosomal aberration assay, CHO cells were exposed to SHetA2 at concentrations 6.8–1750 μg/ml for 3 h in the presence of S9 and 3 h and 21 h in the absence of S9. Cytotoxicity was observed at 109.4 μg/ml for 3 h in the presence of S9, 6.8 μg/ml for 3 h, and 2.5 μg/ml for 21 h in the absence of S9. In the chromosomal aberrations experiments, cells were analyzed at 16, 32, and 64 μg/ml for 3 h in the presence of S9, at 1, 2, and 4 μg/ml for 3 h, and at 0.4, 0.8, and 1.6 μg/ml for 21 h in the absence of S9. The results are shown in Fig. 2. In the presence of S9 at 3 h, there was neither any significant increase in chromosomal aberrations nor was there any significant dose-related increase (2.5% at 16 μg/ml, 2.5% at 32 μg/ml, and 4% at 64 μg/ml compared with 1.5% in controls). The results were reproducible in the second experiment (1.5% at 16 μg/ml, 2.0% at 32 μg/ml, and 1.5% at 64 μg/ml compared with 2.0% in controls). In the absence of S9 at 3 h, there was no significant increase in chromosomal aberrations and there was no dose-related increase, and the frequency of chromosomal aberrations was 2%, at all concentrations (1, 2, and 4 μg/ml), compared with 1.5% in controls. In the absence of S9 at 21 h, the frequency of chromosomal aberrations was 3%, 2%, and 2.5% at 0.4, 0.8, and 1.6 μg/ml respectively, compared with 2% in controls. There was no statistically significant increase in chromosomal aberrations at any dose level and there was no dose-related increase. The increase in cells with chromosomal aberrations for positive controls, for MMS 50 μg/ml and CP 12.5 μg/ml in the first experiment, and MMS 25 μg/ml and CP 12.5 μg/ml in the second experiment, were all significant, with 34%, 30%, 18.4%, and 32% respectively. No dose-related increase in polyploidy occurred under any tested conditions (Table 8).

In the mouse bone marrow micronucleus experiment, male and female mice were exposed to single administrations of 330, 660, or 1,320 mg/kg SHetA2, and the bone marrow was evaluated for cytotoxicity and micronucleus formation. There was no significant suppression of PCE among RBC in either sex at any dose level. The results on MN frequencies are presented in Table 6. No statistically significant increase in the frequency of micronucleated PCE was seen at any dose level in either male or female mice at the 24 or 48 h time points, with one exception: in male mice given 660 mg/kg SHetA2 and sacrificed at 24 h, there was a significant ($p<0.05$) increase in micronucleus frequency (0.25%) compared with the controls (0.13%). However, this increase was within two standard deviations of the mean historical control value (mean + 2 SD = 0.38%). Also, there was no statistically significant dose-related increase in the frequency of micronuclei in either sex at any time point.

3.3. Phospho-ibuprofen (PI)

In the Ames test, a range-finding experiment was conducted with strain TA100 over doses ranging from 156.3–5,000 μg/plate in the presence and absence of S9. Cytotoxicity was seen only at doses ≥ 2500 μg/plate in the absence of S9. The first experiment for mutagenicity was conducted with all five tester strains over the same range of doses, 156.3–5,000 μg/plate, in the presence or absence of S9. The results are presented in Table 4. No statistically significant increase in the number of revertant colonies was seen with any of the strains except for strain TA1537 at 156.3 and 5,000 μg/plate in the presence of S9. These increases were statistically significant ($p < 0.01$); however, they were not dose-dependent. Cytotoxicity was seen with strains TA1535 and TA100 at doses ≥5,000 μg/plate, only in the absence of S9. The second experiment for mutagenicity was conducted over doses 39.1–2,500 μg/plate in the presence or absence of S9. No statistically significant increase in the number of revertant colonies was seen for any of the strains. No cytotoxicity was observed. Because the increases observed in the first experiment with TA1537 were < 2-fold over solvent control values, within the historical spontaneous range for the strain, not dose-dependent and not repoducible, they were not considered to be biologically significant.

In the chromosome aberration assay, a cytotoxicity experiment was conducted from 1–5,000 μg/ml in the presence of S9 for 3 h exposure and in the absence of S9 for 3 and 21 h exposure. In the absence of S9, cytotoxicity (<50% confluency or a >50% reduction in mitotic index) was observed at ≥100 μg/ml for 3 and 21 h exposures. In the absence of S9, cells were tested with doses ranging from 3.125–50 μg/ml for 3 h in the first experiment, and 3.125–25 μg/ml for 21 h in the second experiment. The top three scorable concentrations (6.25, 12.5, and 25 μg/ml for 3 h exposure, and 3.125, 6.25, and 12.5 μg/ml for 21 h exposure) were analyzed. The results are presented in Fig. 3. In the absence of S9 at 3 h exposure, the frequencies of chromosomal aberrations were 3.5%, 3.0%, and 2.5% at 6.25, 12.5, and 25 μg/ml, respectively, compared with 1.5% in controls. In the absence of S9 at 21 h exposure, the frequencies of chromosomal aberrations were 3.0%, 1.5%, and 1.5% at 3.125, 6.25, and 12.5 μg/ml, respectively, compared with 1.0% in controls. There was no statistically significant increase in chromosomal aberration at any concentration for 3 h or 21 h exposures when compared with controls. Positive control values for MMS 50 μg/ml and CP 12.5 μg/ml in the first experiment, and MMS 30 μg/ml and CP 12.5 μg/ml in the second experiment, were all significant with 33.3%, 40%, 40%, and 42% cells with aberrations, respectively. There was also no dose-related increase in polyploidy in the presence and absence of S9 (Table 8).

In the presence of S9, cytotoxicity was observed at ≥ 1000 μg/ml. Cells were treated with doses ranging from 25–800 μg/ml in the first chromosome aberration experiment. Cultures <200 μg/ml had 76–100% confluency while cultures ≥200 μg/ml had inadequate cell growth. The top three scorable concentrations of 25, 50, and 100 μg/ml were analyzed in the first experiment. The results are presented in Fig. 3. In the presence of S9 at 3 h exposure,

the frequencies of chromosomal aberrations were 12.0% at 25 μg/ml, 13.5% at 50 μg/ml, and 19.0% at 100 μg/ml, compared with 2.0% in controls. The type of aberrations were mainly chromatid and chromosome deletions and exchanges. All concentrations had statistically significant ($p < 0.05$) increases in the number of chromosomal aberrations compared with control, and the response was a significant dose-related increase (Z=5.02, $p<0.001$). In the second experiment, a dose range of 3.125–100 μg/ml was tested and all doses were analyzed. The frequencies of chromosomal aberrations were 8.5% at 3.125 μg/ml, 7.0% at 6.25 μg/ml, 5.0% at 12.5 μg/ml, 12.5% at 25 μg/ml, 9.0% at 50 μg/ml, and 10.5% at 100 μg/ml, compared with 2.5% in controls. All concentrations had statistically significant ($p <0.05$) increases in the number of chromosomal aberrations compared with control except for 12.5 μg/ml, and the response was dose-related (Z=2.37, $p<0.01$), indicating a positive response for PI in the presence of S9. No dose-related increase in polyploidy was observed in the presence of S9 (Table 8).

In the mouse bone marrow micronucleus experiment, male and female mice were exposed to a single administration of PI at dose levels of 500, 1000, and 2000 mg/kg, sacrificed 24 or 48 hr later, and their bone marrow were then evaluated for cytotoxicity and micronucleus formation. There was no significant suppression of PCE among RBC in either sex at any dose level. The micronucleus frequencies are presented in Table 7. No statistically significant increase in the frequency of micronucleated PCE was seen at any dose level in either male or female mice at the 24 or 48 hr time point.

3.4. Ibuprofen, phospho-ibuprofen (PI) and diethoxyphosphoryloxybutyl alcohol (DEPBA) in CHO cell cultures

This study was performed to evaluate the ability of phospho-ibuprofen (PI) to induce chromosomal aberrations in CHO cells, and to determine if damage was present, if it had arisen from structural chromosomal aberrations (kinetochore negative, clastogen), or from whole chromosome non-disjunction (kinetochore positive, aneugen) in the presence of S9. For comparison, ibuprofen was used as a reference control. DEPBA, a component in PI was also tested for the induction of chromosomal aberrations and micronuclei.

3.4.1. Chromosomal aberration assay—The results are presented in Fig. 4. *Phospho-ibuprofen (PI).* In the chromosomal aberration experiments, cells were treated with PI at concentrations of 25, 50, and 100 μg/ml for 3 h in the presence of S9. The frequencies of chromosomal aberrations for cultures exposed to PI were 5.5% at 25 μg/ml, 6% at 50 μg/ml, and 9.0% at 100 μg/ml, compared with 2.0% in controls. The type of aberrations were mainly chromatid and chromosome deletions and exchanges. The 50 and 100 μg/ml concentrations had statistically significant ($p < 0.05$) increases in the number of chromosomal aberrations compared with control, and the response was dose-related (Z = 2.93, $p < 0.01$). *Ibuprofen*. For cells exposed to ibuprofen at three scorable concentrations, the frequencies of chromosomal aberrations were 2.5%, 3.5%, and 3.5% at 62.5, 125, and 250 μg/ml, respectively, compared with 2.0% in controls. No statistically significant increase was observed for any concentration. In addition, no dose-related increase in chromosomal aberration was observed. *DEPBA*. Cells were treated with diethoxyphosphoryloxybutyl alcohol (DEPBA) between 13.3–5,000 μg/ml for 3 h in the presence of S9. The frequencies of chromosomal aberrations for cultures exposed to DEPBA were 2.5% at 13.3 μg/ml, 3.0% at 26.5 μg/ml, 1.5% at 53 μg/ml, 2.0% at 500 μg/ml, and 2.0% at 5000 μg/ml compared with 2.5% in the control. There was no statistically significant increase in the number of chromosomal aberration compared with control and there was no dose-related response. For the second experiment, the frequencies of chromosomal aberrations were 2.5%, 3.5%, 3.5%, 4.0%, and 4.0% at 13.3, 26.5, 53, 500, and 5000 μg/ml, respectively, compared with 4.5% in the control. Again, no statistically

significant increase was observed for any concentration. In addition, no dose-related increase in chromosomal aberration was observed. There was no dose-related increase in polyploidy in cultures treated with PI, Ibuprofen or DEPBA (Table 9).

3.4.2. In vitro micronucleus assay with antikinetochore antibody labeling—The results are presented in Fig. 5. *Phospho-ibuprofen.* CHO cells were exposed to PI at concentrations of 25, 50, and 100 μg/ml in the presence of S9 for 3 h exposure. The frequencies of binucleate cells with micronuclei were 3.5% at 25 μg/ml, 4.0% at 50 μg/ml, and 5.7% at 100 μg/ml, compared with 1.7% in controls. All concentrations had statistically significant ($p < 0.05$) increases in the number of micronucleated binucleate cells compared with control, and the response was dose-related (Z=6.90, $p<0.001$). These results were reproducible. The frequencies of micronucleated binucleate cells with kinetochores was 1.6% at 25 μg/ml, 1.9% at 50 μg/ml, and 2.6% at 100 μg/ml, compared with 0.9% in controls. These increases were statistically significant when compared with control at all concentrations, and they were dose–related (Z = 4.35, $p < 0.001$), which indicates the aneugenic potential of the drug. The frequencies of micronucleated binucleate cells without kinetochores were 1.8% at 25 μg/ml, 2.1% at 50 μg/ml, and 3.1% at 100 μg/ml, compared with 0.8% in controls. These increases, at all concentrations, were statistically significant when compared with control, and were dose–related (Z = 5.29, $p < 0.001$), which indicates clastogenic potential. *Ibuprofen.* Cells were exposed to ibuprofen at concentrations 31.25–500 μg/ml in the presence of S9 for 3 h exposure. The frequencies of binucleate cells with micronuclei at scorable concentrations were 1.8% at 62.5 μg/ml, 2.7% at 125 μg/ml, and 4.3% at 250 μg/ml, compared with 1.7% in controls. All concentrations except for 62.5 μg/ml had statistically significant increases ($p < 0.05$) in the number of micronucleated binucleate cells compared with control, and the response was dose-related (Z = 5.96, $p < 0.001$). The frequencies of micronucleated binucleate cells with kinetochores were 0.9% at 62.5 μg/ml, 1.2% at 125 μg/ml, and 2.3% at 250 μg/ml, compared with 0.9% in controls. Only the 250 μg/ml concentration was statistically significant when compared with control, however, the increase was dose-related (Z = 4.44, $p < 0.001$). This indicates that ibuprofen shows a significant aneugenic activity only at the high concentrations. The frequencies of micronucleated binucleate cells without kinetochores were 0.9% at 62.5 μg/ml, 1.5% at 125 μg/ml, and 2.1% at 250 μg/ml, compared with 0.8% in controls. All of the concentrations except for 62.5 μg/ml were statistically significant when compared with control and this increase was dose–related (Z = 3.93, $p < 0.001$), which indicates a clastogenic response induced by ibuprofen at these concentrations. *DEPBA.* CHO cells were exposed to DEPBA between concentrations 13.3–5,000 μg/ml in the presence of S9 for 3 h exposure. The frequencies of binucleate cells with micronuclei were 0.97% at 13.3 μg/ml, 0.98% at 26.5 μg/ml, 1.12% at 53 μg/ml, 1.32% at 500 μg/ml, and 1.16% at 5000 μg/ml, compared with 1.17% in the control in the first experiment. None of the concentrations had any statistically significant increases in the number of micronucleated binucleate cells compared with control, and no dose-related response was observed (Z= 0.31). In the second experiment, the frequencies of micronuclei were 1.62% at 13.3 μg/ml, 1.45% at 26.5 μg/ml, 1.64% at 53 μg/ml, 1.29% at 500 μg/ml, and 1.33% at 5000 μg/ml, compared with 1.47% in control. None of the concentrations had any statistically significant increases in the number of micronucleated binucleate cells compared with control and no dose-related response was observed. Based on these observations, PI was found to induce a significant number of both aneugenic and clastogenic events. In comparison, ibuprofen, the parent compound, induced significant increases in the number of aneugenic events at the high concentration of 250 μg/ml and a significant number of clastogenic events at 125 and 250 μg/ml. DEPBA was negative for aneugenic and clastogenic effects.

3.5. The GreenScreen HC assay

PI was tested in the range of 0.12–31.25 μg/ml; ibuprofen, 0.49–125 μg/ml; and DEPBA, 3.91–1,000 μg/ml, in the presence or absence of S9. Results are presented in Table 8. PI ibuprofen and DEPBA were determined to be cytotoxic without S9 at 3.91 μg/ml, 125 μg/ml and 1,000 μg/ml, respectively. All three compound were non-genotoxic in the presence or absence of S9.

4. Discussion

CP-31398 was non-mutagenic in the Ames test. There was induction of neither chromosomal aberrations or polyploidy in CHO cells nor micronuclei in bone marrow cells of mice after exposure to CP-31398, suggesting that the compound is neither clastogenic nor aneugenic. Several studies have shown anticarcinogenic effects of CP-31398 in various cancers, through the stabilization of p53 and inhibition of p53 binding to DNA. Mutations that inactivate p53, indicative of aggressiveness, exist in over 50% of cancers [27; 28; 29]. p53 is directly involved in DNA damage repair, cell cycle arrest, and apoptosis via transcriptional regulation of genes but also interacts with other proteins to promote the same processes [30; 31]. Because CP-31398 stabilizes p53, it acts as a protectant against thermal denaturation and maintains the monoclonal antibody 1650 epitope conformation in newly synthesized p53 [32]. CP-31398 is able to restore the folding of mutated p53 to a native conformation, permitting wild-type functionality [33] and allow mutant p53 to bind to p53 response elements *in vivo*, as measured by the chromatin immunoprecipitation assay [34]. By inhibiting MdM2-mediated ubiquitination and degradation, CP-31398 stabilizes wild-type p53 in cells [35]. Anticarcinogenic effects of CP-31398 have been observed in UVB-induced skin carcinogenesis in mice and chemically induced colon carcinogenesis in rats [5; 6]. Apoptosis induced by CP-31398 occurred with translocation of p53 to mitochondria, leading to altered mitochondrial membrane potential, cytochrome c release, and reactive oxygen species release. CP-31398 decreased the growth of tumor xenografts composed of wild-type or mutant p53 tumor cells, increasing tumor-free host survival [4]. In addition to the anti-cancer potential of CP-31398, it was negative for genotoxicity in the present study and has high potential as a non-genotoxic anti-cancer compound.

SHetA2 was negative in all three genetic toxicology assays tested. The differential effects of SHetA2 on cancer versus normal cells were associated with direct targeting of the mitochondria and decrease of Bcl-2 and Bcl-x1 proteins in cancer cells, with little effect on normal cells [36]. The complex response of gene expression patterns in endometrial organotypic cultures treated with carcinogens and SHetA2 was modeled with a system biology approach, in which normal endometrial cultures exposed to the carcinogen, DMBA, developed a cancerous phenotype in the absence but not in the presence of SHetA2 [37]. In addition to this chemotherapeutic effect, the lack of genotoxicity observed in this study shows that SHetA2 may be a potential clinical utility for chemoprevention.

Phospho-ibuprofen (PI) was non-mutagenic in the Ames test and was not clastogenic or aneugenic in the *in vivo* mouse bone marrow assay. In the absence of S9, no induction of chromosomal aberrations with PI was observed; however, PI showed a positive response for the induction of structural chromosomal aberrations in the presence of S9. In addition, PI also induced kinetochore negative and positive micronuclei in the presence of S9 which indicates the clastogenic as well as the aneugenic nature of this compound. These results were compared with the parent compound of PI, ibuprofen, and its moiety DEPBA, neither of which induced chromosomal aberrations in the presence of S9. Induction of binucleated cells with micronuclei (BNMN) was observed only at highest concentrations of ibuprofen while no induction of BNMN was observed for DEPBA in the presence of S9. In the presence of rat S9, PI undergoes hydrolysis and oxidation at the 1- and 3-positions of the

isobutyl group, leading to three major metabolites, ibuprofen, 1-OH-PI and carboxyl-PI, and three minor metabolites, 1-OH-ibuprofen, 2-OH–ibuprofen, and carboxyl-ibuprofen [38]. Differential regioselectivity of the oxidation of ibuprofen and PI causes PI to generate 15.6 times more 1-OH-ibuprofen than ibuprofen in murine plasma (2.5 versus 0.16%) [38]. These metabolites may play a major role in inducing chromosomal aberrations in CHO cells exposed to PI in the presence of rat S9 in the present study. However, the induction of micronuclei, which was not observed in bone marrow erythrocytes of mice exposed to PI may indicate that metabolites generated from PI may not be genotoxic in mice and/or may be further metabolized and detoxified [38]. The detoxication of these metabolites varies from species to species; hence, the *in vitro* genotoxic effect may not be translated to *in vivo*. PI inhibits the growth of human colon cancer cells *in vitro* and SW 480 human colon cancer xenografts in nude mice. PI induces oxidative stress only in tumors, and its apoptotic effect was restricted to xenografts. PI acts against cancer through a mechanism distinct from that of various conventional chemotherapeutic drugs, underscoring the critical role of oxidative stress in their effect, and indicating that the pathways leading to oxidative stress may be critical targets for anticancer strategies [39]. Based on these chemoprevention abilities and lack of chromosomal damage in mice, PI may be a potential chemopreventive agent. In addition, PI, Ibuprofen and DEPBA were negative for genotoxicity in the GADD45α-GFP GreenScreen HC assay in the presence and absence of S9, an indication that these compounds are most likely negative in *in vivo* tests [26]. Our study confirms the negative results for the induction of MN in the bone marrow of mice exposed to PI.

In conclusion, CP-31398, SHetA2 and PI were negative in the *Salmonella-Escherichia coli*/microsome plate incorporation test and *in vitro* CHO chromosomal aberrations assay, with one exception. PI exhibited a positive response in the presence of S9, which may indicate that the metabolites generated by PI could be major contributors in inducing chromosomal aberrations in CHO cell cultures. None of the compounds induced micronuclei in mouse bone marrow erythrocytes, which indicates that these drug candidates were not clastogenic or aneugenic in mice and that they may be potential chemopreventive agents for further development and human clinical trials.

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References

1. Kapetanovic IM. Rapid access to preventive intervention development program in the Division of Cancer Prevention of the U.S. National Cancer Institute: an overview. Cancer Epidemiol Biomarkers Prev. 2009; 18:698–700. [PubMed: 19240231]
2. Wang W, Kim SH, El-Deiry WS. Small-molecule modulators of p53 family signaling and antitumor effects in p53-deficient human colon tumor xenografts. Proc Natl Acad Sci U S A. 2006; 103:11003–11008. [PubMed: 16835297]
3. Wischhusen J, Naumann U, Ohgaki H, Rastinejad F, Weller M. CP-31398, a novel p53-stabilizing agent, induces p53-dependent and p53-independent glioma cell death. Oncogene. 2003; 22:8233–8245. [PubMed: 14614447]
4. Xu J, Timares L, Heilpern C, Weng Z, Li C, Xu H, Pressey JG, Elmets CA, Kopelovich L, Athar M. Targeting wild-type and mutant p53 with small molecule CP-31398 blocks the growth of rhabdomyosarcoma by inducing reactive oxygen species-dependent apoptosis. Cancer Res. 2010; 70:6566–6576. [PubMed: 20682800]

5. Rao CV, Swamy MV, Patlolla JM, Kopelovich L. Suppression of familial adenomatous polyposis by CP-31398, a TP53 modulator, in APCmin/+ mice. Cancer Res. 2008; 68:7670–7675. [PubMed: 18794156]
6. Tang X, Zhu Y, Han L, Kim AL, Kopelovich L, Bickers DR, Athar M. CP-31398 restores mutant p53 tumor suppressor function and inhibits UVB-induced skin carcinogenesis in mice. J Clin Invest. 2007; 117:3753–3764. [PubMed: 18060030]
7. Benbrook DM, Kamelle SA, Guruswamy SB, Lightfoot SA, Rutledge TL, Gould NS, Hannafon BN, Dunn ST, Berlin KD. Flexible heteroarotinoids (Flex-Hets) exhibit improved therapeutic ratios as anti-cancer agents over retinoic acid receptor agonists. Invest New Drugs. 2005; 23:417–428. [PubMed: 16133793]
8. Baron JA. Aspirin and NSAIDs for the prevention of colorectal cancer. Recent Results Cancer Res. 2009; 181:223–229. [PubMed: 19213572]
9. Crew KD, Neugut AI. Aspirin and NSAIDs: effects in breast and ovarian cancers. Curr Opin Obstet Gynecol. 2006; 18:71–75. [PubMed: 16493264]
10. Yunis JJ, Lewandowski RC. High-resolution cytogenetics. Birth Defects Orig Artic Ser. 1983; 19:11–37. [PubMed: 6309269]
11. Yunis JJ, Tanzer J. Molecular mechanisms of hematologic malignancies. Crit Rev Oncog. 1993; 4:161–190. [PubMed: 8420572]
12. Bonassi S, Norppa H, Ceppi M, Stromberg U, Vermeulen R, Znaor A, Cebulska-Wasilewska A, Fabianova E, Fucic A, Gundy S, Hansteen IL, Knudsen LE, Lazutka J, Rossner P, Sram RJ, Boffetta P. Chromosomal aberration frequency in lymphocytes predicts the risk of cancer: results from a pooled cohort study of 22 358 subjects in 11 countries. Carcinogenesis. 2008; 29:1178–1183. [PubMed: 18356148]
13. Peters S, Portengen L, Bonassi S, Sram R, Vermeulen R. Intra- and interindividual variability in lymphocyte chromosomal aberrations: implications for cancer risk assessment. Am J Epidemiol. 2011; 174:490–493. [PubMed: 21652601]
14. I.C.o.H.T.G. Recommended for Adoption at Step 4 of the ICH process on July by the ICH Steering Committee (Final Draft). 1997. ICH, A Standard Battery for Genotoxicity Testing of Pharmaceuticals.
15. Cimino MC. Comparative overview of current international strategies and guidelines for genetic toxicology testing for regulatory purposes. Environ Mol Mutagen. 2006; 47:362–390. [PubMed: 16649190]
16. Mortelmans K, Riccio ES. The bacterial tryptophan reverse mutation assay with Escherichia coli WP2. Mutat Res. 2000; 455:61–69. [PubMed: 11113467]
17. Mortelmans K, Zeiger E. The Ames Salmonella/microsome mutagenicity assay. Mutat Res. 2000; 455:29–60. [PubMed: 11113466]
18. Doppalapudi RS, Riccio ES, Rausch LL, Shimon JA, Lee PS, Mortelmans KE, Kapetanovic IM, Crowell JA, Mirsalis JC. Evaluation of chemopreventive agents for genotoxic activity. Mutat Res. 2007; 629:148–160. [PubMed: 17387038]
19. Galloway SM, Bloom AD, Resnick M, Margolin BH, Nakamura F, Archer P, Zeiger E. Development of a standard protocol for in vitro cytogenetic testing with Chinese hamster ovary cells: comparison of results for 22 compounds in two laboratories. Environ Mutagen. 1985; 7:1–51. [PubMed: 3967632]
20. Savage JR. Classification and relationships of induced chromosomal structual changes. J Med Genet. 1976; 13:103–122. [PubMed: 933108]
21. Eastmond DA, Tucker JD. Kinetochore localization in micronucleated cytokinesis-blocked Chinese hamster ovary cells: a new and rapid assay for identifying aneuploidy-inducing agents. Mutat Res. 1989; 224:517–525. [PubMed: 2685592]
22. Hayashi M, Sofuni T, Ishidate M Jr. An application of Acridine Orange fluorescent staining to the micronucleus test. Mutat Res. 1983; 120:241–247. [PubMed: 6855792]
23. MacGregor JT, Heddle JA, Hite M, Margolin BH, Ramel C, Salamone MF, Tice RR, Wild D. Guidelines for the conduct of micronucleus assays in mammalian bone marrow erythrocytes. Mutat Res. 1987; 189:103–112. [PubMed: 3657826]
24. Schmid W. The micronucleus test. Mutat Res. 1975; 31:9–15. [PubMed: 48190]

25. Hastwell PW, Chai LL, Roberts KJ, Webster TW, Harvey JS, Rees RW, Walmsley RM. High-specificity and high-sensitivity genotoxicity assessment in a human cell line: validation of the GreenScreen HC GADD45a-GFP genotoxicity assay. Mutat Res. 2006; 607:160–175. [PubMed: 16781187]
26. Jagger C, Tate M, Cahill PA, Hughes C, Knight AW, Billinton N, Walmsley RM. Assessment of the genotoxicity of S9-generated metabolites using the GreenScreen HC GADD45a-GFP assay. Mutagenesis. 2009; 24:35–50. [PubMed: 18787182]
27. Hollstein M, Rice K, Greenblatt MS, Soussi T, Fuchs R, Sorlie T, Hovig E, Smith-Sorensen B, Montesano R, Harris CC. Database of p53 gene somatic mutations in human tumors and cell lines. Nucleic Acids Res. 1994; 22:3551–3555. [PubMed: 7937055]
28. Lane DP, Hupp TR. Drug discovery and p53. Drug Discov Today. 2003; 8:347–355. [PubMed: 12681938]
29. Soussi T, Beroud C. Assessing TP53 status in human tumours to evaluate clinical outcome. Nat Rev Cancer. 2001; 1:233–240. [PubMed: 11902578]
30. Vousden KH. p53: death star. Cell. 2000; 103:691–694. [PubMed: 11114324]
31. Willis AC, Chen X. The promise and obstacle of p53 as a cancer therapeutic agent. Curr Mol Med. 2002; 2:329–345. [PubMed: 12108946]
32. Foster BA, Coffey HA, Morin MJ, Rastinejad F. Pharmacological rescue of mutant p53 conformation and function. Science. 1999; 286:2507–2510. [PubMed: 10617466]
33. Rippin TM, Bykov VJ, Freund SM, Selivanova G, Wiman KG, Fersht AR. Characterization of the p53-rescue drug CP-31398 in vitro and in living cells. Oncogene. 2002; 21:2119–2129. [PubMed: 11948395]
34. Demma MJ, Wong S, Maxwell E, Dasmahapatra B. CP-31398 restores DNA-binding activity to mutant p53 in vitro but does not affect p53 homologs p63 and p73. J Biol Chem. 2004; 279:45887–45896. [PubMed: 15308639]
35. Luu Y, Bush J, Cheung KJ Jr, Li G. The p53 stabilizing compound CP-31398 induces apoptosis by activating the intrinsic Bax/mitochondrial/caspase-9 pathway. Exp Cell Res. 2002; 276:214–222. [PubMed: 12027451]
36. Liu T, Masamha CP, Chengedza S, Berlin KD, Lightfoot S, He F, Benbrook DM. Development of flexible-heteroarotinoids for kidney cancer. Mol Cancer Ther. 2009; 8:1227–1238. [PubMed: 19417155]
37. Benbrook DM, Lightfoot S, Ranger-Moore J, Liu T, Chengedza S, Berry WL, Dozmorov I. Gene expression analysis of biological systems driving an organotypic model of endometrial carcinogenesis and chemoprevention. Gene Regul Syst Bio. 2008; 2:21–42.
38. Xie G, Sun Y, Nie T, Mackenzie GG, Huang L, Kopelovich L, Komninou D, Rigas B. Phospho-ibuprofen (MDC-917) is a novel agent against colon cancer: efficacy, metabolism, and pharmacokinetics in mouse models. J Pharmacol Exp Ther. 2011; 337:876–886. [PubMed: 21422165]
39. Sun Y, Huang L, Mackenzie GG, Rigas B. Oxidative stress mediates through apoptosis the anticancer effect of phospho-nonsteroidal anti-inflammatory drugs: implications for the role of oxidative stress in the action of anticancer agents. J Pharmacol Exp Ther. 2011; 338:775–783. [PubMed: 21646387]

Highlights

- CP31398, SHetA2 and PI were negative in the Ames test and in the mouse bone marrow micronucleus test.
- CP31398 and SHetA2 were negative for chromosomal aberrations (CA) in CHO cells.
- PI was negative in the absence of S9 and positive in the presence of S9 for CA in CHO cells.
- Induction of CA with PI in the presence of S9 may be due to the metabolites generated in cultures.
- All 3 compounds may be potential chemopreventive agents for further development.

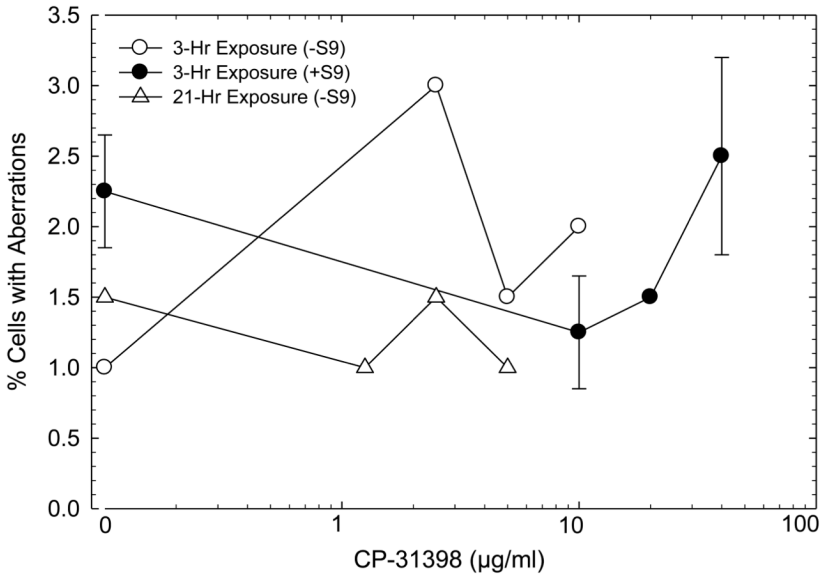

Fig. 1.
Induction of chromosomal aberrations in CHO cells treated with CP-31398

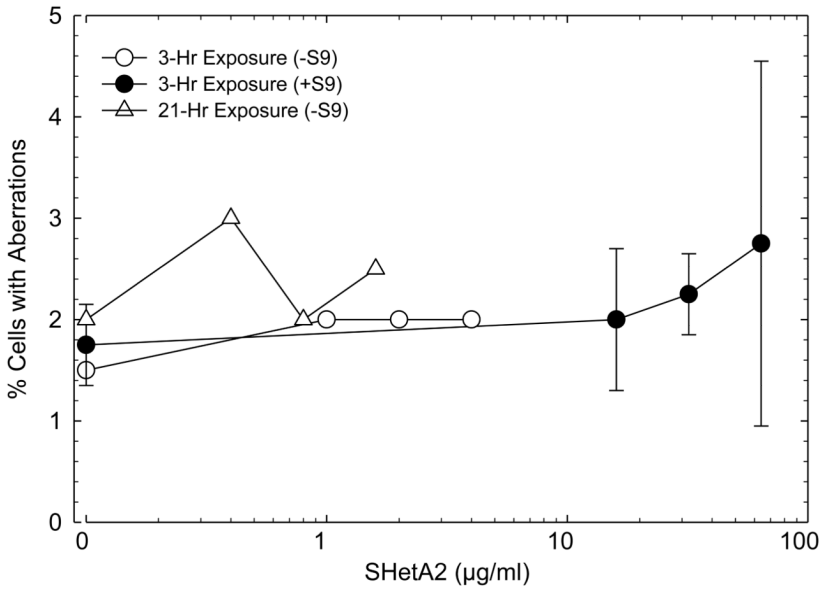

Fig. 2.
Induction of chromosomal aberrations in CHO cells treated with SHetA2

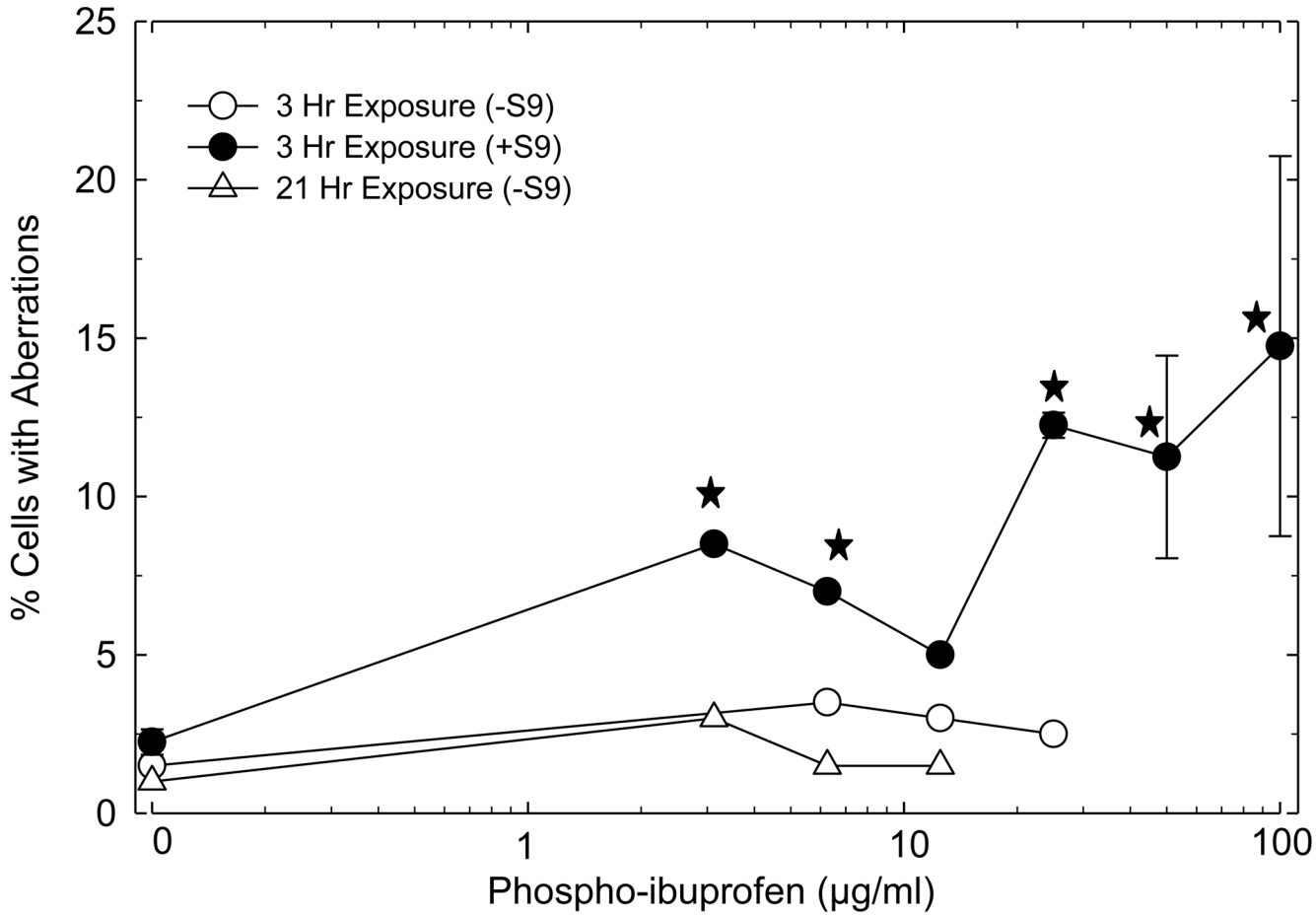

Fig. 3.
Induction of chromosomal aberrations in CHO cells treated with PI
Stars indicate that the increase in chromosomal aberrations was significant compared with controls. *(P<0.05*)

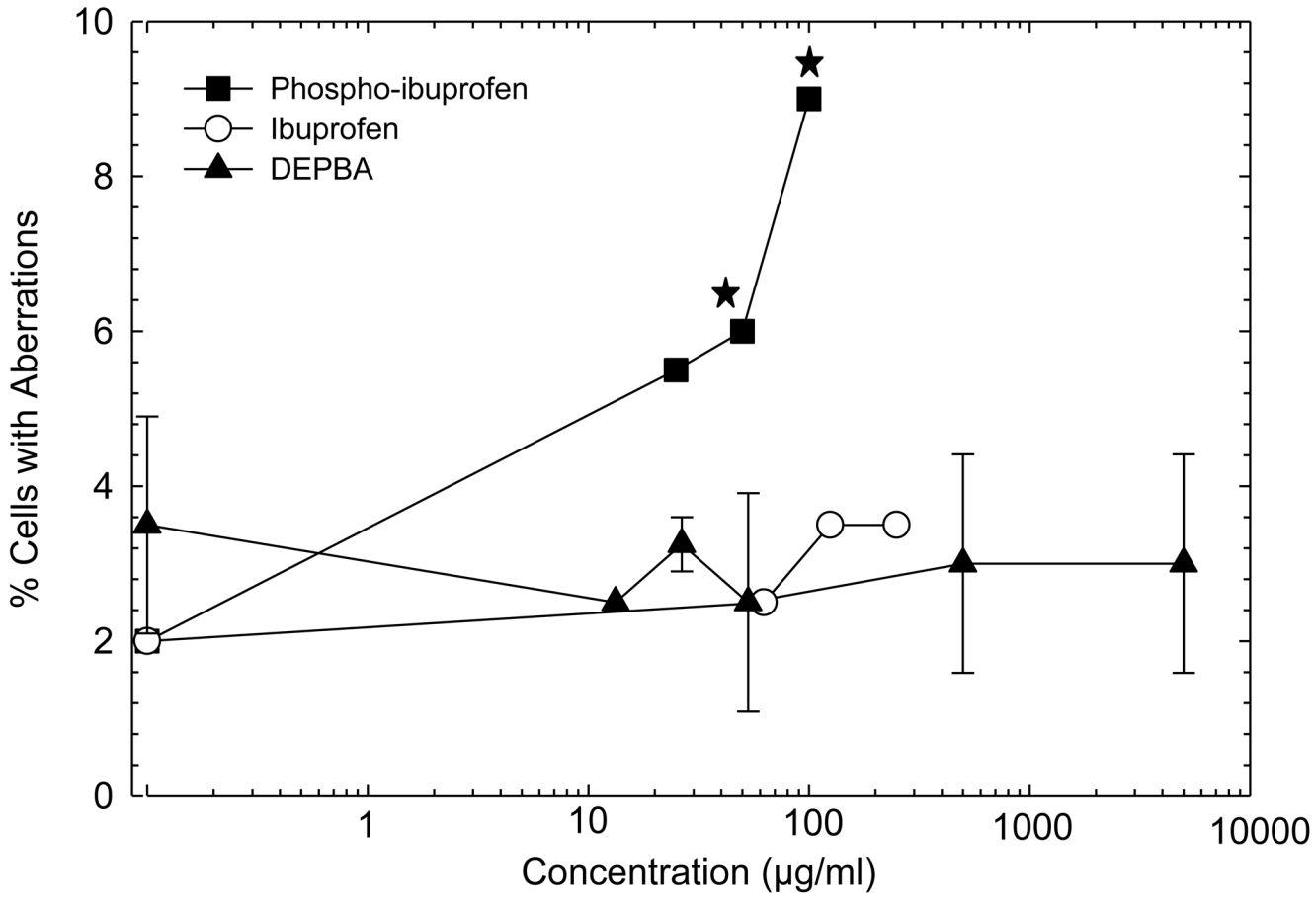

Fig. 4.

Induction of chromosomal aberrations treated with PI, Ibuprofen and DEPBA in the presence of S9

Stars indicate that the increase in chromosomal aberrations was significant compared with controls. *(P<0.05*)

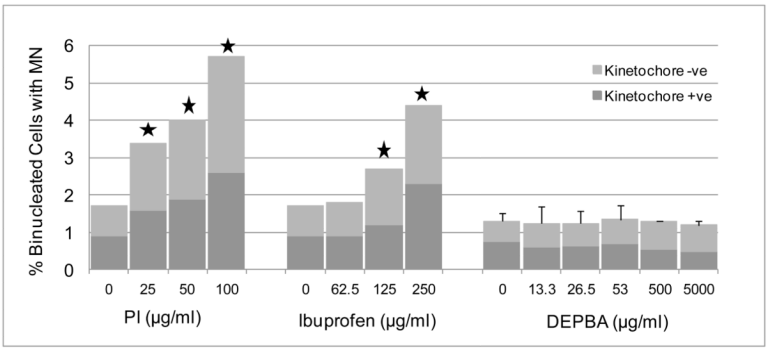

Fig 5.

Induction of micronuclei in CHO cells treated with PI, Ibuprofen and DEPBA in the presence of S9

Stars indicate that the increase in micronuclei was significant compared with controls. *(P<0.05*)

Table 1

Overview on the genotoxicity of tested compounds.

Compound	Test				
	Ames Test	**CHO-CA**	**CHO-MN**	**Mouse BMMN**	**Green Screen Assay**
CP-31398	Negative	Negative	Not done	Negative	Not done
SHetA2	Negative	Negative	Not done	Negative	Not done
Phospho-ibuprofen	Negative	Negative without S9 Positive with S9	Positive with S9	Negative	Negative
Ibuprofen	Not done	Negative with S9	Positive with S9	Not done	Negative
DEPBA	Not done	Negative with S9	Negative with S9	Not done	Negative

CHO-CA = Chromosomal aberration study in Chinese Hamster Ovary cells; CHO-MN = Micronucleus study in Chinese Hamster Ovary cells; Mouse BMMN = Mouse bone marrow micronucleus study; S9 = rat hepatic S9 metabolic activation

Mutat Res. Author manuscript; available in PMC 2013 July 04.

Table 2

Effect of CP-31348 on bacterial strains in the Ames test.

Mean ± Standard Deviation Revertants/Plate						Mean ± Standard Deviation Revertants/Plate					
Dose (μg)/Plate	**TA1535**	**TA1537**	**TA98**	**TA100**	**Wp*uvr* A**	**Dose (μg)/Plate**	**TA1535**	**TA1537**	**TA98**	**TA100**	**Wp*uvr* A**
Without S9						**Without S9**					
0	14 ± 2	11 ± 1	33 ± 10	148 ± 2	26 ± 4	0	19 ± 6	11 ± 5	29 ± 2	141 ± 14	27 ± 4
78.1	21 ± 4	12 ± 5	32 ± 3	158 ± 20	23 ± 4	39.1	14 ± 3	11 ± 4	26 ± 1	144 ± 15	31 ± 8
156.3	12 ± 4	15 ± 4	32 ± 9	176 ± 6	25 ± 6	78.1	9 ± 3	10 ± 2	31 ± 8	146 ± 23	30 ± 5
312.5	14 ± 5	12 ± 6	31 ± 5	168 ± 22	24 ± 6	156.3	15 ± 3	14 ± 3	24 ± 6	139 ± 10	31 ± 3
625	13 ± 2	13 ± 3	23 ± 0	92 ± 14	16 ± 2	312.5	16 ± 0	7 ± 3	29 ± 7	140 ± 29	34 ± 4
1250	4 ± 1	6 ± 1	14 ± 3	20 ± 13	11 ± 1	625	10 ± 2	4 ± 3	26 ± 1	84 ± 19	20 ± 6
2500	CT	CT	CT	CT	CT	1250	3 ± 2	CT	7 ± 5	CT	14 ± 4
						2500	CT	CT	CT	CT	CT
With S9 (5%)						**With S9 (10%)**					
0	14 ± 4	8 ± 6	42 ± 4	159 ± 11	30 ± 7	0	17 ± 6	12 ± 2	34 ± 1	167 ± 18	35 ± 4
78.1	15 ± 5	17 ± 3	46 ± 6	188 ± 18	30 ± 3	39.1	11 ± 6	9 ± 6	36 ± 4	175 ± 9	33 ± 5
156.3	15 ± 4	16 ± 2	50 ± 9	199 ± 12	33 ± 5	78.1	10 ± 5	11 ± 5	32 ± 5	162 ± 18	36 ± 5
312.5	16 ± 6	7 ± 3	49 ± 7	192 ± 16	31 ± 4	156.3	17 ± 3	13 ± 3	38 ± 4	202 ± 5 *	37 ± 3
625	8 ± 5	10 ± 5	36 ± 5	113 ± 19	27 ± 4	312.5	16 ± 2	10 ± 2	37 ± 3	207 ± 5 *	39 ± 9
1250	1 ± 1	2 ± 1	15 ± 6	40 ± 18	21 ± 6	625	7 ± 3	8 ± 3	33 ± 10	165 ± 4	27 ± 3
2500	CT	CT	CT	CT	CT	1250	2 ± 3	1 ± 1	15 ± 4	31 ± 29	13 ± 4
						2500	CT	CT	CT	CT	CT
Experiment 1						Experiment 2					

* $p < 0.01$; CT = Cytotoxic.

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Table 3

Effect of SHetA2 on bacterial strains in the Ames test

Mean ± Standard Deviation Revertants/Plate						Mean ± Standard Deviation Revertants/Plate						
Dose (μg)/Plate	**TA1535**	**TA1537**	**TA98**	**TA100**	**Wp*uvr* A**	**Dose (μg)/Plate**	**TA1535**	**TA1537**	**TA98**	**TA100**	**Dose (μg)/Plate**	**Wp*uvr* A**
Without S9						**Without S9**						
0	15 ± 1	8 ± 3	33 ± 5	129 ± 27	23 ± 3	0	18 ± 1	13 ± 2	19 ± 6	134 ± 13	0	20 ± 1
0.31	20 ± 5	5 ± 0	41 ± 3	136 ± 14	20 ± 2	0.31	14 ± 3	10 ± 3	23 ± 4	125 ± 12	2.44	18 ± 4
0.61	15 ± 3	10 ± 2	33 ± 7	140 ± 6	28 ± 2	0.61	16 ± 5	10 ± 2	29 ± 6	141 ± 8	4.88	16 ± 5
1.22	10 ± 2	8 ± 2	37 ± 4	157 ± 13	21 ± 4	1.22	19 ± 6	13 ± 1	25 ± 2	150 ± 12	9.77	17 ± 3
2.44	10 ± 4	5 ± 1	34 ± 3	130 ± 6	23 ± 4	2.44	20 ± 2	13 ± 2	27 ± 2	148 ± 8	19.53	19 ± 3
4.88	CT	3 ± 2	22 ± 6	72 ± 17	27 ± 3	4.88	5 ± 2	2 ± 1	7 ± 2	56 ± 13	39.06	21 ± 2
9.77	CT	1 ± 1	5 ± 3	25 ± 29	20 ± 1	9.77	CT	CT	CT	CT	78.13	12 ± 2
19.53	CT	CT	CT	14 ± 1	19 ± 6						156.3	9 ± 3
With S9 (5%)						**With S9 (10%)**						
0	14 ± 1	7 ± 2	48 ± 7	145 ± 23	28 ± 2	0	15 ± 1	9 ± 2	34 ± 1	132 ± 24	0	20 ± 3
0.31	13 ± 3	11 ± 2	37 ± 2	144 ± 4	26 ± 6	1.22	15 ± 3	4 ± 1	35 ± 2	133 ± 10	2.44	16 ± 2
0.61	17 ± 1	13 ± 2	38 ± 5	134 ± 18	26 ± 6	2.44	14 ± 4	12 ± 1	39 ± 5	144 ± 3	4.88	19 ± 2
1.22	16 ± 2	12 ± 3	48 ± 8	148 ± 20	21 ± 2	4.88	14 ± 4	12 ± 1	41 ± 6	156 ± 3	9.77	23 ± 3
2.44	13 ± 2	8 ± 3	43 ± 3	150 ± 9	30 ± 2	9.77	13 ± 6	12 ± 4	40 ± 2	135 ± 14	19.53	22 ± 3
4.88	9 ± 4	11 ± 1	45 ± 10	154 ± 14	30 ± 2	19.53	11 ± 0	11 ± 4	39 ± 6	166 ± 21	39.06	20 ± 1
9.77	9 ± 3	12 ± 4	51 ± 8	157 ± 4	23 ± 6	39.06	5 ± 2	CT	44 ± 8	162 ± 22	78.13	19 ± 3
19.53	5 ± 1	5 ± 3	53 ± 11	166 ± 8	27 ± 4	78.13	CT	CT	35 ± 2	26 ± 2	156.3	9 ± 1
Experiment 1						Experiment 2						

CT = Cytoxic.

Table 4

Effect of Phospho-ibuprofen on bacterial strains in the Ames test

Mean ± Standard Deviation Revertants/Plate						Mean ± Standard Deviation Revertants/Plate					
Dose (μg)/Plate	**TA1535**	**TA1537**	**TA98**	**TA100**	**Wp*uvr* A**	**Dose (μg)/Plate**	**TA1535**	**TA1537**	**TA98**	**TA100**	**Wp*uvr* A**
Without S9						**Without S9**					
0	20 ± 2	12 ± 2	18 ± 7	108 ± 8	18 ± 2	0	11 ± 4	11 ± 1	25 ± 7	126 ± 7	29 ± 7
156.3	13 ± 2	6 ± 3	26 ± 2	94 ± 4	17 ± 2	39.1	11 ± 4	9 ± 5	29 ± 9	141 ± 16	22 ± 6
312.5	8 ± 1	7 ± 1	18 ± 2	81 ± 19	18 ± 3	78.1	16 ± 1	6 ± 2	25 ± 2	119 ± 11	24 ± 9
625	6 ± 1	4 ± 1	22 ± 4	93 ± 7	20 ± 2	156.3	12 ± 4	7 ± 2	30 ± 1	109 ± 15	24 ± 2
1250	12 ± 5	7 ± 4	18 ± 5	85 ± 4	15 ± 3	312.5	11 ± 1	8 ± 4	25 ± 6	111 ± 10	20 ± 2
2500	16 ± 2	5 ± 1	16 ± 2	86 ± 1	21 ± 5	625	9 ± 2	9 ± 4	21 ± 4	115 ± 21	28 ± 1
5000	8 ± 6	5 ± 3	15 ± 4	73 ± 13	12 ± 3	1250	14 ± 4	4 ± 1	27 ± 2	118 ± 8	20 ± 3
						2500	13 ± 3	8 ± 3	20 ± 5	101 ± 8	17 ± 2
With S9 (5%)						**With S9 (10%)**					
0	11 ± 4	8 ± 1	26 ± 7	139 ± 25	22 ± 4	0	9 ± 1	13 ± 2	21 ± 4	149 ± 12	32 ± 4
156.3	6 ± 2	14 ± 2 *	25 ± 5	114 ± 13	28 ± 2	39.1	13 ± 3	9 ± 2	26 ± 6	159 ± 39	26 ± 3
312.5	11 ± 7	10 ± 3	25 ± 5	93 ± 12	23 ± 4	78.1	17 ± 6	15 ± 5	23 ± 6	155 ± 12	32 ± 8
625	11 ± 5	9 ± 1	26 ± 4	109 ± 8	22 ± 4	156.3	13 ± 3	9 ± 3	31 ± 8	146 ± 7	36 ± 7
1250	7 ± 4	9 ± 1	22 ± 3	100 ± 5	27 ± 2	312.5	13 ± 5	10 ± 3	39 ± 9	155 ± 9	29 ± 4
2500	12 ± 4	9 ± 2	24 ± 5	101 ± 12	29 ± 3	625	10 ± 2	7 ± 3	25 ± 4	140 ± 11	33 ± 4
5000	9 ± 2	13 ± 1 *	21 ± 4	88 ± 6	21 ± 3	1250	12 ± 4	7 ± 2	36 ± 3	141 ± 9	31 ± 7
						2500	10 ± 2	10 ± 2	29 ± 2	115 ± 21	28 ± 7
Experiment 1						Experiment 2					

* $p < 0.01$

Table 5

Frequency of micronuclei in the bone marrow of mice treated with CP-31398

Treatment	Sex	Dose (mg/kg)	Time in Hours	n	PCE/RBC (%) Mean ± S.E.	PCE with MN (%) Mean ± S.E.
Control	Male	0	24	5	51.85 ± 0.89	0.15 ± 0.03
CP-31398	Male	250	24	5	49.73 ± 1.11	0.13 ± 0.03
CP-31398	Male	500	24	5	47.21 ± 1.59	0.15 ± 0.02
CP-31398	Male	1000	24	4	47.97 ± 0.67	0.14 ± 0.05
Urethane	Male	300	24	5	47.11 ± 0.70	2.36 ± 0.30**
Control	Female	0	24	5	49.83 ± 1.59	0.12 ± 0.03
CP-31398	Female	250	24	5	51.16 ± 1.05	0.06 ± 0.02
CP-31398	Female	500	24	5	53.78 ± 1.62	0.11 ± 0.04
CP-31398	Female	1000	24	5	52.16 ± 0.99	0.14 ± 0.05
Control	Male	0	48	4	44.18 ± 4.94	0.11 ± 0.02
CP-31398	Male	250	48	5	48.96 ± 0.75	0.04 ± 0.02
CP-31398	Male	500	48	5	46.91 ± 2.52	0.06 ± 0.02
CP-31398	Male	1000	48	4	46.82 ± 2.71	0.16 ± 0.04
Control	Female	0	48	5	55.43 ± 3.50	0.07 ± 0.01
CP-31398	Female	250	48	5	51.24 ± 1.06	0.12 ± 0.03
CP-31398	Female	500	48	5	52.39 ± 2.75	0.14 ± 0.01
CP-31398	Female	1000	48	5	50.29 ± 2.56	0.05 ± 0.02

n = Number of surviving animals; PCE = Polychromatic erythrocytes; RBC= Red blood cells; MN = Micronuclei; Control = 0.5% Methyl Cellulose;

* = $p < 0.05$ by test for binomial proportions for test article;

** = $p < 0.01$ by test for binomial proportions for positive control

Table 6

Frequency of micronuclei in the bone marrow of mice treated with SHetA2

Treatment	Sex	Dose (mg/kg)	Time in Hours	n	PCE/RBC (%) Mean ± S.E.	PCE with MN (%) Mean ± S.E.
Control	Male	0	24	5	48.89 ± 0.71	0.13 ± 0.02
SHetA2	Male	330	24	5	50.23 ± 1.12	0.18 ± 0.06
SHetA2	Male	660	24	5	49.93 ± 0.99	0.25 ± 0.06 *
SHetA2	Male	1320	24	5	35.18 ± 6.83	0.23 ± 0.04
Urethane	Male	300	24	5	49.95 ± 1.62	3.28 ± 0.30 **
Control	Female	0	24	5	51.87 ± 1.97	0.15 ± 0.03
SHetA2	Female	330	24	5	53.18 ± 1.65	0.09 ± 0.02
SHetA2	Female	660	24	5	49.62 ± 0.87	0.10 ± 0.03
SHetA2	Female	1320	24	5	49.59 ± 1.16	0.11 ± 0.02
Control	Male	0	48	5	47.40 ± 2.41	0.14 ± 0.04
SHetA2	Male	330	48	5	51.64 ± 2.86	0.12 ± 0.03
SHetA2	Male	660	48	5	53.50 ± 1.89	0.11 ± 0.04
SHetA2	Male	1320	48	5	50.07 ± 0.55	0.18 ± 0.04
Control	Female	0	48	5	48.87 ± 1.03	0.14 ± 0.04
SHetA2	Female	330	48	5	53.63 ± 1.58	0.13 ± 0.04
SHetA2	Female	660	48	5	48.19 ± 0.90	0.11 ± 0.02
SHetA2	Female	1320	48	5	47.01 ± 2.24	0.17 ± 0.04

n = Number of surviving animals; PCE = Polychromatic erythrocytes; RBC= Red blood cells; MN = Micronuclei; Control = 0.5% Methyl Cellulose;

* = $p < 0.05$ by test for binomial proportions for test article;

** = $p < 0.01$ by test for binomial proportions for positive control

Table 7

Frequency of micronuclei in the bone marrow of mice treated with phospho-ibuprofen

Treatment	Sex	Dose (mg/kg)	Time in Hours	n	PCE/RBC (%) Mean ± S.E.	PCE with MN (%) Mean ± S.E.
Control	Male	0	24	5	47.46 ± 3.03	0.15 ± 0.03
Phospho-Ibuprofen	Male	500	24	5	52.60 ± 2.42	0.10 ± 0.03
Phospho-Ibuprofen	Male	1000	24	5	49.02 ± 2.63	0.10 ± 0.02
Phospho-Ibuprofen	Male	2000[A]	24	4	49.94 ± 2.33	0.21 ± 0.06
Urethane	Male	300	24	5	51.10 ± 2.39	2.17 ± 0.26**
Control	Female	0	24	5	48.54 ± 1.64	0.12 ± 0.04
Phospho-Ibuprofen	Female	500	24	5	44.84 ± 1.74	0.13 ± 0.05
Phospho-Ibuprofen	Female	1000	24	5	50.10 ± 0.53	0.15 ± 0.02
Phospho-Ibuprofen	Female	2000	24	5	53.44 ± 4.86	0.20 ± 0.02
Control	Male	0	48	5	48.33 ± 1.57	0.14 ± 0.02
Phospho-Ibuprofen	Male	500	48	5	50.95 ± 2.77	0.04 ± 0.02
Phospho-Ibuprofen	Male	1000	48	5	52.31 ± 2.86	0.12 ± 0.03
Phospho-Ibuprofen	Male	2000	48	5	46.94 ± 2.55	0.18 ± 0.01
Control	Female	0	48	5	49.61 ± 2.76	0.11 ± 0.04
Phospho-Ibuprofen	Female	500	48	5	51.92 ± 3.84	0.18 ± 0.05
Phospho-Ibuprofen	Female	1000	48	5	46.36 ± 1.53	0.11 ± 0.03
Phospho-Ibuprofen	Female	2000	48	5	46.95 ± 0.91	0.08 ± 0.03

[A] = One male mouse in this group found dead before necropsy; n = Number of surviving animals; PCE = Polychromatic erythrocytes; RBC= Red blood cells; MN = Micronuclei; Control = PEG 400;

* = $p < 0.05$ by test for binomial proportions for test article;

** = $p < 0.01$ by test for binomial proportions for positive control

Table 8

Polyploidy index in CHO cells treated with CP31398, SHetA2 or Phospho-ibuprofen

Exposure	CP-31398		SHetA2		Phospho-ibuprofen	
	Concentration μg/ml	Polyploidy Index (%)	Concentration μg/ml	Polyploidy Index (%)	Concentration μg/ml	Polyploidy Index (%)
3 Hr −S9	Sterile Water	0.5	DMSO	0.9	DMSO	1.4
	2.5	1.0	1	0.9	6.25	0.5
	5	0.0	2	0.0	12.5	1.4
	10	0.5	4	0.9	25	1.4
	MMS, 50 μg/ml	0.5	MMS, 50 μg/ml	0.5	MMS, 50 μg/ml	0.4
3 Hr +S9	Sterile Water	0.5	DMSO	0.0	DMSO	0.5
	10	0.0	16	0.0	25	0.9
	20	0.5	32	1.0	50	1.4
	40	0.5	64	0.0	100	0.9
	CP, 12.5 μg/ml	0.0	CP, 12.5 μg/ml	0.0	CP, 12.5 μg/ml	0.5
21 Hr −S9	Sterile Water	0.0	DMSO	0.0	DMSO	1.0
	1.25	1.5	0.4	0.0	3.125	0.5
	2.5	1.0	0.8	1.0	6.25	1.5
	5	0.0	1.6	0.0	12.5	1.0
	MMS, 20 μg/ml	0.0	MMS, 25 μg/ml	0.0	MMS, 30 μg/ml	0.5
3 Hr +S9	Sterile Water	0.5	DMSO	1.4	DMSO	1.9
	10	1.5	16	0.9	3.125	1.0
	20	0.0	32	1.4	6.25	1.5
	40	1.5	64	0.0	12.5	0.5
	CP, 12.5 μg/ml	0.0	CP, 12.5 μg/ml	0.0	25	0.5
					50	1.0
					100	1.9
					CP, 12.5 μg/ml	0.5

DMSO= Dimethyl sulfoxide; MMS= Methyl methanesulfonate; CP= Cyclophosphamide; S9 = Rat hepatic metabolic activation.

Table 9

Polyploidy index in Chinese hamster ovary cells treated with DEPBA, Ibuprofen and PI

	DEPBA		Ibuprofen & PI	
Exposure	**Concentration μg/ml**	**Polyploidy Index (%)**	**Concentration μg/ml**	**Polyploidy Index (%)**
3 Hr +S9	DMSO	2.0	DMSO	0.9
	13.3	1.5	Ibuprofen, 62.5	0.5
	26.5	2.0	Ibuprofen, 125	1.4
	53	1.5	Ibuprofen, 250	1.4
	500	2.4	PI, 25	0.5
	5000	0.9	PI, 50	1.9
	CP, 12.5 μg/ml	0.0	PI, 100	1.4
			CP, 9 μg/ml	0.5
3 Hr +S9	DMSO	1.0		
	13.3	1.0		
	26.5	3.0		
	53	0.5		
	500	2.0		
	5000	1.5		
	CP, 12.5 μg/ml	0.5		

DMSO= Dimethyl sulfoxide; CP= Cyclophosphamide; S9 = rat hepatic S9 metabolic activation. PI= Phospho-ibuprofen

Table 10

GADD45α-GFP GreenScreen HC assay results

Test Article	Cytotoxicity Results Without Activation (LEC)	Genotoxicity Results without Activation (LEC)	Genotoxicity Results with Activation (LEC)
Phospho-ibuprofen	3.91 μg/ml	Non-genotoxic	Non-genotoxic
Ibuprofen	125 μg/ml	Non-genotoxic	Non-genotoxic
DEPBA	1000 μg/ml	Non-genotoxic	Non-genotoxic

LEC = Lowest Effective Concentration