Improvement of the Therapeutic Index of Anticancer Drugs by the Superoxide Dismutase Mimic Mangafodipir

Jérôme Alexandre, Carole Nicco, Christiane Chéreau, Alexis Laurent, Bernard Weill, François Goldwasser, Frédéric Batteux

Background: Anticancer drugs act by increasing intracellular hydrogen peroxide levels. Mangafodipir, a superoxide dismutase (SOD) mimic with catalase and glutathione reductase activities, protects normal cells from apoptosis induced by H₂O₂. We investigated its and other oxidative stress modulators’ effects on anticancer drug activity in vitro and in vivo.

Methods: Cell lysis and intracellular reactive oxygen species levels were assessed in vitro in human leukocytes from healthy subjects and in murine CT26 colon cancer cells. Cells were exposed to the chemotherapeutic agents paclitaxel, oxaliplatin, or 5-fluorouracil, either in the presence or absence of mangafodipir and other oxidative stress modulators. Cell viability was evaluated by the methylthiazolotetrazolium assay. The effects of mangafodipir and other oxidative stress modulators on peripheral blood counts and on tumor growth were studied in BALB/c mice that were implanted with CT26 tumors and treated with 20 mg/kg paclitaxel. Survival of BALB/c mice infected with Staphylococcus aureus was also examined by treatment group. Statistical tests were two-sided.

Results: In vitro lysis of leukocytes exposed to paclitaxel, oxaliplatin, or 5-fluorouracil in combination with mangafodipir was decreased by 46% (95% confidence interval [CI] = 44% to 48%), 30.5% (95% CI = 29% to 32%), and 15% (95% CI = 10% to 20%), compared with lysis of cells treated with anticancer agent alone. Mangafodipir also statistically significantly enhanced in vitro anticancer drug cytotoxicity toward CT26 cancer cells. In vivo, mangafodipir protected mice against paclitaxel-induced leukopenia. Moreover, the survival rate of mice infected with S. aureus and treated with paclitaxel was higher when mangafodipir was also administered (survival: 3 of 17 versus 14 of 17, P<.001). In addition, mangafodipir amplified the inhibitory effect of paclitaxel on CT26 tumor growth in mice.


Reactive oxygen species, such as superoxide anions, and hydrogen peroxide (H₂O₂) are normal byproducts of cellular aerobic metabolism. Superoxide anions (O₂⁻) are secondarily converted to H₂O₂ by the superoxide dismutases (SODs). H₂O₂ has a dual role in cellular homeostasis. Low levels of intracellular H₂O₂ stimulate cell growth (1), whereas high levels of H₂O₂ lead to cell senescence and apoptosis (2,3). Several anticancer agents, such as 5-fluorouracil (4), platinum (5,6), arsenic trioxide (7), paclitaxel (8), and anthracyclines (9,10) increase levels of reactive oxygen species. Tumor cells have higher levels of reactive oxygen species than normal cells and are therefore more sensitive to the additional oxidative stress generated by anticancer agents (5,6,11).

Cells also contain a variety of free radical scavenging systems that protect them from the effects of drugs that generate oxygen free radicals. Catalase and glutathione peroxidase are enzymes that detoxify H₂O₂. High levels in tumor cells of reduced glutathione, the cofactor of glutathione peroxidase, have been associated with a multidrug resistance phenotype (12). Recent studies have suggested that compounds that target such protective mechanisms could enhance the activity of anticancer agents and reverse the multidrug resistance phenotype. For example, buthionine sulfoximine (BSO), a glutathione synthesis inhibitor, can increase the cytotoxicity of melphalan by preventing glutathione peroxidase activity and increasing H₂O₂ levels (13). However, BSO depletes glutathione in both normal and cancer cells, which increases melphalan’s hematologic toxicity and prevents any enhancement of the therapeutic index of this anticancer agent (14,15). Conversely, N-acetylcysteine protects normal cells from the cytotoxic effects of anticancer drugs by preventing elevation of intracellular H₂O₂ through its catalase- and glutathione reductase–like properties (6).

SOD enzymes can also affect tumor cell proliferation via their effects on peroxide levels. Indeed, overexpression of SOD in human cancer cell lines increases H₂O₂ production and reduces tumor growth in the absence of anticancer agents (16,17). We have previously reported that two nonpeptidyl mimics of SOD, copper [II] diisopropylsalicylate (CuDIPS) and manganese [III] tetrakis-(5,10,15,20)-benzoic acid porphyrin (MnTBAP), increase the cytotoxic activity of anticancer drugs by increasing H₂O₂ levels (6). In addition, mangafodipir, a contrast agent used in magnetic resonance imaging, can protect normal liver cells against reactive oxygen species–induced apoptosis (18). Mangafodipir is a chelate of manganese [II] and of the ligand fodipir, a vitamin B6 derivate. Mangafodipir has SOD-, catalase-, and glutathione reductase–like properties, allowing it to act at multiple steps of the reactive oxygen species cascade.
We hypothesized that the association of those properties in a single molecule such as mangafodipir could be beneficial through its ability to enhance the killing of cancer cells while protecting normal cells from chemotherapeutic toxicity. More specifically, we investigated the ability of mangafodipir to minimize the hematologic toxicity and potentiate the antitumor activity of several commonly used anticancer agents both in vitro and in vivo. The effects of mangafodipir were compared with those of CuDIPS, a Cu/ZnSOD mimic with no catalase or glutathione reductase activity (19); MnTBAP, a MnSOD mimic with catalase activity (20); and N-acetylcysteine, an antioxidant molecule that has both catalase and glutathione-reductase activities but not SOD activity (21), in tumor cell lines, in human leukocytes, and in mice.

**METHODS**

**Chemicals and Cell Lines**

All chemicals were from Sigma (Saint Quentin Fallavier, France), except for mangafodipir (Teslascan; Amersham Health, Amersham, U.K.), oxaliplatin (Eloxatine; Sanofi-Aventis, Paris, France), docetaxel (Taxotere; Sanofi-Aventis), and paclitaxel (Taxol; Bristol Myers Squibb, Rueil Malmaison, France), for which commercial solutions were used. CT26 (mouse colon carcinoma, American Type Culture Collection [ATCC] 2638), Hepa1.6 (mouse liver hepatoma, ATCC 1830), and A549 (human lung carcinoma, ATCC CCL-185) cell lines were obtained from ATCC (Manassas, VA). Tumor cells were cultured in Dulbecco’s Modified Eagle’s medium—Glutamax-I (Life Technologies, Cergy Pontoise, France) supplemented with 10% heat-inactivated fetal calf serum (Life Technologies) and antibiotics. All cell lines were routinely tested to ensure that they were free of Mycoplasma infection.

**Human Leukocytes**

Human leukocytes were obtained from six healthy volunteers and 10 cancer patients. All cancer patients (eight women and two men) were diagnosed with metastatic or locally advanced solid tumors (breast \(n = 5\), prostate \(n = 2\), lung \(n = 1\), stomach \(n = 1\), and ovary \(n = 1\)) and were part of a pharmacokinetic study of docetaxel reported elsewhere (22). They had given written informed consent to an additional blood collection for the purpose of the present study. The study was approved by the Ethics Committee of Hopital Cochin, Paris. Ten milliliters of blood were collected before any antitumor treatment, hemolyzed with 0.15 mM ammonium chloride, and then centrifuged at 1800 rpm (700g) for 5 minutes. The pellet was suspended in RPMI medium—Glutamax-I supplemented with 10% heat-inactivated fetal calf serum and antibiotics (complete medium). Leukocytes (\(5 \times 10^4\) cells/well) were then seeded in 96-well plates (Costar Corning, Inc., Corning, NY) and immediately exposed to anticancer agents and/or to oxidative stress modulators.

**In Vitro Cell Viability Assays**

Tumor cells (\(2 \times 10^4\) cells/well) or human leukocytes from healthy volunteers (\(5 \times 10^4\) cells/well) were seeded in 96-well plates and incubated for 24 hours in complete medium (DMEM or RPMI, respectively) with the anticancer agent (paclitaxel, oxaliplatin, or 5-fluorouracil) alone or with varying amounts of oxidative stress modulators (mangafodipir, CuDIPS, MnTBAP, or N-acetylcysteine). Tumor cells and leukocytes from healthy volunteers were incubated with 10 and 20 \(\mu\)M paclitaxel, 10 \(\mu\)M and 1 \(m\)M oxaliplatin, or 50 \(\mu\)M and 40 \(m\)M 5-fluorouracil, respectively. Human leukocytes from cancer patients were incubated with 20, 30, and 40 \(\mu\)M docetaxel alone or with 400 \(\mu\)M mangafodipir. Cell viability was evaluated by the mitochondrion-dependent reduction of methylthiazol tetrazolium (MTT) to formazan. In brief, cells were exposed to 50 \(\mu\)L of MTT (2 mg/mL in phosphate-buffered saline [PBS]) and incubated for 4 hours at 37 °C. MTT reduction, which indicates viable cells, was visualized by addition of 100 \(\mu\)L dimethyl sulfoxide. The absorbance at 550 nm in each well was recorded with an enzyme-linked immunosorbent assay (ELISA) microplate reader (VICTOR2; Perkin Elmer, Paris, France). Results are expressed as percentage of viable cells compared with untreated cells (which have 100% viability).

**In Vivo Hematologic Toxicity Analysis**

BALB/c female mice between 6 and 8 weeks of age (Iffa Credo, L’Arbresles, France) were used in all experiments. Mice received humane care in compliance with institutional guidelines. PBS (1.54 mM potassium phosphate monobasic, 155.17 mM sodium chloride, 2.71 mM sodium phosphate dibasic; Life Technologies) at pH 7.2 was used as the vehicle. Mice were injected intraperitoneally with vehicle alone (group 1); with paclitaxel, mangafodipir, MnTBAP, CuDIPS, or N-acetylcysteine alone (groups 2 to 6); or with paclitaxel and one of the oxidative stress modulators in combination (groups 7 to 10). Five mice were treated in each group. Paclitaxel (20 mg/kg) or vehicle alone was administered on days 0, 2, and 4. This dose of paclitaxel was determined in previous experiments (6) and was used because it was associated with a 50% reduction in tumor volume of CT26 at day 15 without any substantial clinical toxicity. On days 0, 2, 4, and 7, mice were administered 10 mg/kg of mangafodipir, MnTBAP, or CuDIPS, 150 mg/kg of N-acetylcysteine, or vehicle. Ten days after the first injection of paclitaxel or oxidative stress modulator, mice were killed by cervical dislocation. Spleen, bone marrow from femoral bone, and 1 mL of blood were then collected. The total number of hematopoietic cells in bone marrow and leukocytes from spleen and blood were counted using a Malassez cell.

**Susceptibility of Mice to Bacterial Infection**

In another experiment, BALB/c female mice were injected intraperitoneally with either vehicle alone (group 1), paclitaxel (60 mg/kg) alone on days 0, 3, and 6 (group 2), or paclitaxel (60 mg/kg) in combination with mangafodipir (10 mg/kg) on days 0, 3, and 6 (group 3). A sublethal dose of the pathogen *Staphylococcus aureus* (200 \(\mu\)L of a stock suspension with an optical density of 600 at 550 nm) was then injected intraperitoneally on day 9. The survival rate of mice was evaluated 48 hours following *S. aureus* injection. A total of 17 mice were treated in each group in three independent experiments.
In Vivo Antitumor Activity

Each BALB/c female mouse was injected subcutaneously in the back of the neck with $2 \times 10^6$ of CT26 cells. When the tumor reached a size of 200–500 mm², mice were grouped in 10 groups so that the sizes of the tumors were not statistically significantly different by group. Mice were injected intraperitoneally with vehicle alone (group 1); with paclitaxel, mangafodipir, MnTBAP, CuDIPS, or N-acetylcysteine alone (groups 2 to 6); or with paclitaxel and one of these oxidative stress modulators in combination (groups 7 to 10). Paclitaxel (20 mg/kg) or vehicle was administered on days 0, 2, and 4. Then 10 mg/kg mangafodipir, MnTBAP, or CuDIPS or 150 mg/kg N-acetylcysteine or vehicle were administered three times a week for 1 month. Ten mice were treated in each group. Tumor size was measured with a Vernier calliper every 3 days at the same time that the oxidative stress modulator was administered. Tumor volume (TV) was calculated as follows: $TV (\text{mm}^3) = (L \times W^2)/2$, where L is the longest and W the shortest radius of the tumor. Results are expressed as the mean of tumor volume within each group. Tumor volumes were compared across treatment groups after each tumor size measurement, i.e., every 3 days.

Intracellular Levels of Superoxide Anions, Hydrogen Peroxide, and Reduced Glutathione

Tumor cells ($2 \times 10^6$/well) or human leukocytes from one healthy volunteer ($5 \times 10^4$/well) were seeded in 96-well plates and incubated for 48 hours in complete medium with anticancer agents (oxaliplatin, paclitaxel, or 5-fluorouracil) alone or in combination with 400 μM mangafodipir, 100 μM CuDIPS, 100 μM MnTBAP, or 400 μM N-acetylcysteine. Tumor cells and leukocytes were incubated with 10 and 20 μM paclitaxel, 10 μM and 1 mM oxaliplatin, or 50 μM and 40 mM 5-fluorouracil, respectively. Levels of intracellular superoxide anion ($O_2^-$) and $H_2O_2$ were assessed spectrofluorometrically (VICTOR2) by oxidation of dihydroethidium (DHE) (Molecular Probes, Leiden, The Netherlands) and of 2′,7′-dichlorodihydrofluorescein diacetate ($H_2DCFDA$) (Molecular Probes), respectively. Cells were washed once with PBS and then incubated with either 250 μM DHE or 200 μM $H_2DCFDA$ in PBS for 1 hour at 37 °C. Excitation and emission wavelengths used were 500 and 605 nm for DHE and 490 and 535 nm for $H_2DCFDA$, respectively. The number of adherent cells was evaluated by the crystal violet assay. In brief, cells were stained in 0.5% crystal violet and 30% ethanol in PBS for 30 minutes at room temperature. After two washes in PBS, the stain was dissolved in 50% ethanol, and absorbance was measured at 560 nm on an ELISA multiwell reader. The levels of $O_2^-$ or $H_2O_2$ were calculated in each sample as follows: reactive oxygen species rate (arbitrary units/min/10⁶ cells) = (fluorescence intensity [arbitrary units] at T₆₀ minutes − fluorescence intensity [arbitrary units] at T₀)/60 minutes/number of adherent cells as measured by the crystal violet assay. Levels of intracellular reduced glutathione were assessed spectrofluorometrically by monochlorobimane staining (23). Briefly, tumor cells ($2 \times 10^6$/well) or human leukocytes ($5 \times 10^4$/well) were seeded in 96-well plates and incubated for 48 hours in complete medium with anticancer agent alone or in combination with an oxidative stress modulator at the concentrations described above. Cells were washed once with PBS and then incubated with 50 μM monochlorobimane diluted in PBS. The fluorescence intensity was measured after 15 minutes at 37 °C. Excitation and emission wavelengths were 380 and 485 nm, respectively. The intracellular glutathione level was expressed as arbitrary units of fluorescence intensity/number of adherent cells as measured by the crystal violet assay.

Statistical Analysis

All values from the in vitro experiments, except for those using leukocytes from cancer patients, are the mean values from three independent experiments, each carried out in triplicate. Data obtained using leukocytes from cancer patients are the mean values from the 10 cancer patients from one experiment carried out in triplicate. Error bars in the figures represent 95% confidence intervals (CIs) and are shown when larger than the symbol. Differences between treated and untreated groups were analyzed by the chi-square test for categorical data and by the Student’s t test for comparison of means. P values less than .05 were considered statistically significant.

Results

In Vitro Effect of Mangafodipir and Other Oxidative Stress Modulators on Human Leukocytes Treated With Anticancer Agents

We first investigated the effect of oxidative stress modulators on anticancer drug–induced cytotoxicity of normal leukocytes. Leukocytes from six healthy volunteers were exposed in vitro to the alkylating agent oxaliplatin, the taxane paclitaxel, or the anti-metabolite 5-fluorouracil at concentrations approaching IC₅₀ values for 24 hours in the presence or absence of mangafodipir. In one representative healthy volunteer (Fig. 1, A–C), concomitant exposure to increasing concentrations of mangafodipir statistically significantly decreased the cytotoxicity of these three anticancer agents in a dose-dependent fashion compared with any of the anticancer agents alone. For example, a concentration of 400 μM mangafodipir led to an absolute increase in cell survival of 46% (95% CI = 44% to 48%), 30.5% (95% CI = 29% to 32%), and 15% (95% CI = 10% to 20%) after 24 hours of exposure to 20 μM paclitaxel, 1 mM oxaliplatin, or 40 mM 5-fluorouracil, respectively, compared with survival of cells treated with each drug alone. N-Acetylcysteine displayed a similar protective effect against the cytotoxicity of paclitaxel, oxaliplatin, and 5-fluorouracil toward human normal leukocytes. By contrast, CuDIPS and MnTBAP statistically significantly enhanced the cytotoxicity of paclitaxel, oxaliplatin, and 5-fluorouracil against leukocytes, although the effects were more pronounced with CuDIPS than with MnTBAP (Fig. 1, A–C). Thus, concomitant treatment with 100 μM CuDIPS or MnTBAP decreased cell survival by 44% (95% CI = 43% to 45%) and 3.6% (95% CI = 2.9% to 4.3%) after exposure to paclitaxel for 24 hours, respectively, compared with survival of cells treated with paclitaxel alone. We also assessed whether mangafodipir was effective in protecting leukocytes from the toxic effects of another taxane, docetaxel. Mangafodipir protected the leukocytes from the 10 cancer patients against the toxicity of docetaxel. Simultaneous incubation for 24 hours with 20 μM docetaxel and 400 μM mangafodipir increased the viability rate by 19.5% (95% CI = 16% to 23%) compared with the rate of cells incubated with docetaxel alone.
experiments carried out in triplicates. Error bars represent 95% confidence intervals and are shown when larger than the symbol.

(C3), and 50 μM (C1), 12.5 μM (C2), 100 μM (C3), and 50 μM (C4). Concentrations of CuDIPS or MnTBAP and 40 mM, respectively) (C and F), in the presence or absence of copper [II] diisopropylsalicylate (CuDIPS) (diamonds), manganese [III] tetrakis-(5,10,15,20)-benzoic acid porphyrin (MnTBAP) (squares), N-acetylcysteine (triangles), or mangafodipir (circles). Concentrations of CuDIPS or MnTBAP were 100 μM (C1), 50 μM (C2), 25 μM (C3), and 12.5 μM (C4). Concentrations of N-acetylcysteine or mangafodipir were 400 μM (C1), 200 μM (C2), 100 μM (C3), and 50 μM (C4). Cell viability was assessed by methylthiazoletetrazolium assay and expressed as mean percent cell survival compared with untreated cells (100% viability). All values are the mean values from three independent experiments carried out in triplicates. Error bars represent 95% confidence intervals and are shown when larger than the symbol.

Fig. 1. Effect of mangafodipir and other oxidative stress modulators on the toxicity of anticancer agents toward normal human leukocytes and murine CT26 colon cancer cells in vitro. Leukocytes from a single healthy volunteer (A–C) and CT26 (D–F) cancer cells were incubated for 24 hours in culture medium with either oxaliplatin (10 μM for tumor cells and 1 mM in leukocytes, respectively) (A and D), paclitaxel (10 and 20 μM, respectively) (B and E), or 5-fluorouracil (5-FU) (50 μM and 40 mM, respectively) (C and F), in the presence or absence of copper [II] diisopropylsalicylate (CuDIPS) (diamonds), manganese [III] tetrakis-(5,10,15,20)-benzoic acid porphyrin (MnTBAP) (squares), N-acetylcysteine (triangles), or mangafodipir (circles). Concentrations of CuDIPS or MnTBAP were 100 μM (C1), 50 μM (C2), 25 μM (C3), and 12.5 μM (C4). Concentrations of N-acetylcysteine or mangafodipir were 400 μM (C1), 200 μM (C2), 100 μM (C3), and 50 μM (C4). Cell viability was assessed by methylthiazoletetrazolium assay and expressed as mean percent cell survival compared with untreated cells (100% viability). All values are the mean values from three independent experiments carried out in triplicates. Error bars represent 95% confidence intervals and are shown when larger than the symbol.

In Vivo Effect of Mangafodipir and Other Oxidative Stress Modulators on Hematologic Toxicity of Paclitaxel and Susceptibility of Mice to Bacterial Infection

We next investigated the effect of mangafodipir and three other oxidative stress modulators in combination with paclitaxel in BALB/c mice. After 10 days of treatment with paclitaxel alone (group 2), a statistically significant decrease in the absolute number of peripheral lymphocytes, neutrophils, and monocytes was observed in blood drawn from treated mice compared with blood drawn from control mice (group 1) (Table 1). This decrease was accompanied by a decrease in total bone marrow cell numbers. Administering mangafodipir (group 7) or N-acetylcysteine (group 10) in combination with paclitaxel abrogated the hematologic toxicity of paclitaxel. Neither mangafodipir nor N-acetylcysteine seemed to enhance hematopoiesis by itself. Indeed, blood and bone marrow cell counts in mice treated with mangafodipir (group 3) or N-acetylcysteine (group 6) alone were similar to those in untreated mice. By contrast to mangafodipir, the two other SOD mimics, CuDIPS (group 8) and MnTBAP (group 9), did not alter the hematologic toxicity of paclitaxel.

Because bacterial infection is the main complication of neutropenia, the susceptibility of mice to S. aureus was tested after administration of paclitaxel alone (group 2) or in combination with mangafodipir (group 3) (Table 2). No deaths were observed in untreated mice inoculated with a sublethal dose of S. aureus. By contrast, 14 of 17 mice died within 48 hours after inoculation with the same dose of S. aureus following treatment with paclitaxel at a dose that induced neutropenia. However, only three of 17 mice inoculated with the bacteria died when mangafodipir had been administered in combination with paclitaxel ($P<.001$).

Effect of Mangafodipir on Cytotoxicity of Anticancer Agents Against CT26 Cancer Cells In Vitro

Mangafodipir, as well as CuDIPS and MnTBAP, statistically significantly increased the cytotoxicity of oxaliplatin (Fig. 1, D), paclitaxel (Fig. 1, E), and 5-fluorouracil (Fig. 1, F) toward mouse CT26 colon cancer cells in a dose-dependent fashion. Conversely, N-acetylcysteine decreased the cytotoxicity of oxaliplatin, 5-fluorouracil, and paclitaxel toward CT26 cancer cells (Fig. 1, D–F). Similar results were observed using murine hepatoma Hepa1.6 and human lung cancer A549 cell lines (data not shown).

Effect of Mangafodipir and Other Oxidative Stress Modulators in Mice Implanted with CT26 Tumor Cells and Treated With Paclitaxel

To determine whether oxidative stress modulators inhibit tumor growth, we next treated mice bearing CT26 tumors with either paclitaxel, mangafodipir, MnTBAP, CuDIPS, or N-acetylcysteine alone or with paclitaxel in combination with one of these oxidative stress modulators (Fig. 2). Mice treated with paclitaxel alone (group 2) developed smaller tumors than untreated mice (group 1), but the difference was statistically significant only at day 27 and not before or after ($\sim 37\%$, 95% CI $= -7\%$ to $-67\%$, $P = .02$ versus untreated mice at day 27). Mangafodipir inhibited tumor growth when administered alone (group 3) to a similar extent as paclitaxel (Fig. 2, A) ($\sim 34\%$, 95% CI $= -2\%$ to $-66\%$ at day 27, $P = .05$ versus untreated mice). Mice treated simultaneously with mangafodipir and paclitaxel (group 7) had statistically significantly smaller tumors than untreated mice at days 24, 27, and 30 ($\sim 49\%$, 95% CI $= -7\%$ to $-91\%$, $P = .04$ at day 27). The concentrations of mangafodipir and paclitaxel slightly amplified the antitumor effect seen with paclitaxel alone, but the difference was not statistically significant. CuDIPS (group 4) and MnTBAP (group 5) had only minimal antitumoral effects when administered alone and did not amplify the antitumoral effect of paclitaxel (Fig. 2, B and C). By contrast, mice treated with N-acetylcysteine alone (group 6) developed larger tumors than untreated mice, but the difference was not statistically significant (Fig. 2, D). Adding N-acetylcysteine to
paclitaxel (group 10) abrogated the antitumor effect of paclitaxel (Fig. 2, D).

Effect of Mangafodipir and Other Oxidative Stress Modulators on Intracellular H2O2 and O2− Generation in Normal and Cancer Cells Exposed to Anticancer Agents In Vitro

We next investigated the effects of mangafodipir and other oxidative stress modulators on levels of reactive oxygen species in cancer cell lines and normal leukocytes. Basal H2O2 and O2− levels were lower in normal human leukocytes than in CT26 cancer cells (Table 3) (*P<.001). Exposure to oxaliplatin, paclitaxel, or 5-fluorouracil for 48 hours statistically significantly increased intracellular levels of O2− and H2O2 in both types of cells. (All comparisons between treated and untreated cells reached P<.001, with the exception of 5-fluorouracil, for which P = .005 and P = .03 for H2O2 and O2− levels, respectively). However, reactive oxygen species levels remained in normal leukocytes than in cancer cells after exposure to these agents (Table 3). Exposure to these anticancer agents for 48 hours also statistically significantly decreased the intracellular reduced glutathione pool by 30% to 40% in both cancer and normal cells compared with untreated cells (both P<.005). Similar results were observed with Hepa1.6 cells (data not shown).

Simultaneous treatment of CT26 cells and human leukocytes with mangafodipir, CuDIPS, or MnTBAP decreased O2− accumulation induced by all three anticancer agents by 20% to 30% in cancer cells (P<.01 compared with cells treated with anticancer agents alone). N-Acetylcysteine did not alter the O2− level in cells treated with anticancer agents.

In CT26 cancer cells, CuDIPS, MnTBAP, and mangafodipir increased H2O2 accumulation (Fig. 3, A–C) and glutathione depletion (Fig. 4, A–C) induced by oxaliplatin, paclitaxel, and 5-fluorouracil. Similar results were obtained when the same set of experiments were conducted in Hepa1.6 cancer cells (data not shown). By contrast, in normal human leukocytes treated with paclitaxel, concomitant exposure to mangafodipir led to a 33% decrease in H2O2 accumulation (95% CI = 29% to 37%, P<.001) compared with normal human leukocytes treated with paclitaxel alone, whereas concomitant exposure to CuDIPS or MnTBAP led to 28% (95% CI = 27% to 29%, P<.001) and 34% (95% CI = 29% to 39%, P<.001) increases in H2O2 accumulation, respectively (Fig. 3, E–G).

Glutathione is an essential cofactor in H2O2 catabolism. Exposure to mangafodipir increased glutathione levels by 45% (95% CI = 40% to 50%, P<.001) in leukocytes also exposed to paclitaxel, whereas CuDIPS and MnTBAP decreased glutathione levels by 15% (95% CI = 10.5% to 19.5%, P<.001) and 26.5% (95% CI = 15% to 38%, P<.001), respectively, when compared with leukocytes exposed to paclitaxel alone (Fig. 4, E–G). Similar effects of mangafodipir were observed in cells treated with oxaliplatin or 5-fluorouracil (Fig. 4, G). Simultaneous treatment with N-acetylcysteine statistically significantly lowered H2O2

Table 1. Hematologic toxicity of paclitaxel administered alone or in combination with oxidative stress modulators in mice*

<table>
<thead>
<tr>
<th>Treatment group†</th>
<th>Spleen</th>
<th>Bone marrow</th>
<th>Total leukocytes</th>
<th>Neutrophils</th>
<th>Lymphocytes</th>
<th>Monocytes</th>
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<tbody>
<tr>
<td>1. Controls</td>
<td>42.2 (33.5 to 50.9)</td>
<td>12.3 (10.6 to 14.0)</td>
<td>7600 (6271 to 8929)</td>
<td>1244 (1155 to 1973)</td>
<td>5612 (4915 to 6309)</td>
<td>424 (320 to 528)</td>
</tr>
<tr>
<td>2. Paclitaxel</td>
<td>44.2 (39.0 to 49.4)</td>
<td>6.7 (5.7 to 7.7)</td>
<td>3400 (2184 to 4616)</td>
<td>222 (128 to 316)</td>
<td>3054 (1950 to 4158)</td>
<td>124 (70 to 178)</td>
</tr>
<tr>
<td>3. Mangafodipir</td>
<td>50.0 (41.6 to 58.4)</td>
<td>13.3 (11.7 to 14.8)</td>
<td>7250 (6491 to 8009)</td>
<td>1528 (1008 to 2047)</td>
<td>5784 (5057 to 6511)</td>
<td>364 (222 to 506)</td>
</tr>
<tr>
<td>4. MnTBAP</td>
<td>47.2 (38.2 to 56.2)</td>
<td>12.0 (10.9 to 13.1)</td>
<td>7800 (6700 to 8900)</td>
<td>1452 (1108 to 1796)</td>
<td>5784 (5057 to 6511)</td>
<td>364 (222 to 506)</td>
</tr>
<tr>
<td>5. CuDIPS</td>
<td>45.6 (35.0 to 56.2)</td>
<td>13.2 (10.8 to 15.6)</td>
<td>7600 (6757 to 8443)</td>
<td>1888 (1460 to 2316)</td>
<td>5376 (4672 to 6808)</td>
<td>336 (156 to 516)</td>
</tr>
<tr>
<td>6. NAC</td>
<td>43.6 (30.7 to 50.7)</td>
<td>12.2 (9.9 to 14.5)</td>
<td>7600 (7120 to 8080)</td>
<td>1564 (1155 to 1973)</td>
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<td>5376 (4672 to 6808)</td>
<td>336 (156 to 516)</td>
</tr>
<tr>
<td>mangafodipir</td>
<td>45.6 (35.0 to 56.2)</td>
<td>13.2 (10.8 to 15.6)</td>
<td>7600 (6757 to 8443)</td>
<td>1888 (1460 to 2316)</td>
<td>5376 (4672 to 6808)</td>
<td>336 (156 to 516)</td>
</tr>
</tbody>
</table>

* MnTBAP = manganese [III] tetrakis-(5,10,15,20)-benzoic acid porphyrin; CuDIPS = copper [II] diisopropylsalicylate; NAC = N-acetylcysteine. Cell numbers are expressed as mean numbers of cells/μL (blood), 106 cells/μL (spleen) or 106 cells/μL (bone marrow), with 95% confidence intervals.
† BALB/c female mice were injected intraperitoneally with 20 mg/kg paclitaxel (on day [D] 0, D2, D4) and/or 10 mg/kg mangafodipir, MnTBAP, or CuDIPS or 150 mg/kg N-acetylcysteine (NAC) (D0, D2, D4, D7). One group of mice remained untreated. Five mice were treated in each group. All mice were killed on day 10 and spleen cells, bone marrow cells, and blood leukocytes were counted.‡ All P values from Student’s t test; comparison group was mice treated with paclitaxel alone.

Table 2. Survival rate of Staphylococcus aureus–injected mice*

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Untreated</td>
<td>5/5</td>
<td>6/6</td>
<td>6/6</td>
<td>17/17</td>
</tr>
<tr>
<td>2. Paclitaxel</td>
<td>0/5</td>
<td>2/6</td>
<td>1/6</td>
<td>3/17</td>
</tr>
<tr>
<td>3. Paclitaxel +</td>
<td>2/5</td>
<td>6/6</td>
<td>6/6</td>
<td>14/17</td>
</tr>
</tbody>
</table>

* BALB/c female mice were injected intraperitoneally with 60 mg/kg paclitaxel (on day [D] 0, D3, D6) alone or in combination with 10 mg/kg mangafodipir (D0, D3, D6). One group of mice remained untreated. A sublethal dose of the pathogen S. aureus was injected intraperitoneally at D9 to all mice, and the survival rate was evaluated at 48 hours. A total of 17 mice were treated in each group in three independent experiments.
accumulation and glutathione depletion induced by all three anticancer agents, both in cancer and normal cells compared with cells treated with anticancer agents alone (Fig. 3, D and H, and Fig. 4, D and H).

**DISCUSSION**

We report what is, to our knowledge, the first evidence that mangafodipir, a molecule that is currently used as a contrast agent for magnetic resonance imaging, has properties that may be useful in cancer therapy. We observed that mangafodipir protected human peripheral blood leukocytes drawn from healthy volunteers and cancer patients from the toxicity of several different anticancer agents that have distinct mechanisms of action. We also observed that mangafodipir protected BALB/c mice treated with paclitaxel from developing leukopenia infections and also amplified the antitumoral effect of paclitaxel toward implanted tumors.

To investigate whether the SOD-, catalase-, and glutathione reductase–like activities of mangafodipir were responsible for its protective action, three other oxidative stress modulators—N-acetylcysteine, CuDIPS, and MnTBAP—were also studied to determine whether they protected leukocytes against toxicity from anticancer agents. In our in vitro experiments, N-acetylcysteine, like mangafodipir, protected normal leukocytes from the cytotoxic effects of paclitaxel, oxaliplatin, and 5-fluorouracil. By contrast, CuDIPS and MnTBAP enhanced the toxicity of these drugs in leukocytes. The absence of a protective effect of the latter two compounds may be due to their lack of glutathione reductase activity, which reduces their capacity to detoxify H$_2$O$_2$. By contrast, the protective effect of mangafodipir and N-acetylcysteine could...
result from the presence of glutathione reductase activity, which limits depletion of glutathione and in turn enables detoxification of H\textsubscript{2}O\textsubscript{2}.

Similar observations were made in vivo in mice treated with the hematoxic anticancer agent paclitaxel. Simultaneous administration of mangafodipir, but not of CuDIPS or MnTBAP, protected mice from paclitaxel-induced hematologic and bone marrow toxicities at day 10. Moreover, coadministration of mangafodipir and paclitaxel dramatically improved the survival rate of mice infected with \textit{S. aureus}. N-Acetylcysteine also had protective effects similar to those of mangafodipir, in agreement with previous data showing that N-acetylcysteine can abrogate the hematologic toxicity of alkylators in mice (24). However, the use of N-acetylcysteine in association with anticancer drugs has been limited by its capacity to accelerate tumor growth, which neutralizes the antitumoral activity of the drugs (6,8).

Other nonpeptidyl SOD mimics have also been shown to decrease the toxicity of anticancer agents or ionizing radiation in normal cells (25–27). For example, M40403, a pure MnSOD mimic, is able to block interleukin-2–induced hypotension in mice, presumably by scavenging extracellular interleukin-2–induced O\textsubscript{2}-, which inactivates circulating catecholamines (25,26). Moreover, in a randomized trial, orgotein, a Cu/ZnSOD mimic that does not cross cell membranes, abrogated the late but not the acute side effects of pelvic irradiation (27). This protective effect of orgotein was attributed to the inhibition of the acute-phase inflammatory events mediated by radiation-induced O\textsubscript{2}-. The two pure SOD mimics explored in these studies prevented specific side effects related to extracellular O\textsubscript{2}- accumulation, whereas in this study, mangafodipir was observed to prevent hematologic toxicity related to intracellular H\textsubscript{2}O\textsubscript{2} accumulation.

One major concern of the finding that mangafodipir prevents hematologic toxicity induced by anticancer agents is the possibility that mangafodipir could abrogate the toxicity of anticancer agents in cancer cells, as it does in normal cells. However, under the same in vitro experimental conditions as above, mangafodipir enhanced the antitumoral activity of the three agents tested. In vivo, mangafodipir abrogated the growth of implanted tumors when administered alone and enhanced the antitumoral effects of paclitaxel in combination. Therefore, mangafodipir may enhance the therapeutic index of anticancer agents by both protecting normal cells and increasing the antitumoral activity of these agents.

The opposing effects of mangafodipir in normal and cancer cells may be related to differences in the redox status of these cells. Indeed, we have confirmed here that the basal concentration of reactive oxygen species and particularly of H\textsubscript{2}O\textsubscript{2} is higher in cancer cells than in normal cells (6). The higher oxidative stress in tumor cells seems to be related to a higher rate of production of superoxide anions by the respiratory chain (6,28) and cytoplasmic NADPH oxidase (29). Furthermore, tumor cells express lower levels of catalase, glutathione peroxidase, reductase, and their respective cofactors than normal cells (6,10,30,31). Because anticancer agents induce the generation of H\textsubscript{2}O\textsubscript{2} in both normal and cancer cells (5,6), the threshold of toxicity is reached more easily in cancer cells than in normal cells (6). This difference leads to preferential inhibition of cell proliferation and increasing cell death in cancer cells.

A limitation of our study is that the antitumoral effect of mangafodipir was studied only in established tumor cell lines. Further studies are warranted to evaluate its activity against primary human tumors. Moreover, relatively high doses of mangafodipir were used in mice compared with those used in the imagery diagnostic setting. Associations between pharmacokinetic and pharmacodynamic parameters need to be evaluated in animals and in humans.
In conclusion, our results support investigation of the use of mangafodipir in cancer patients, because mangafodipir may enhance the therapeutic index of anticancer agents by both protecting normal cells and increasing the antitumoral activity of these agents. The safety of mangafodipir administered as a constant level of anticancer agents plus mangafodipir, CuDIPS, MnTBAP, or NAC was shown in clinical studies to be acceptable in human subjects.

**REFERENCES**


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NOTE

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