Disulfiram inhibits activating transcription factor/cyclic AMP-responsive element binding protein and human melanoma growth in a metal-dependent manner *in vitro*, in mice and in a patient with metastatic disease

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Abstract

The thiocarbamate alcoholism drug disulfiram blocks the P-glycoprotein extrusion pump, inhibits the transcription factor nuclear factor-κB, sensitizes tumors to chemotherapy, reduces angiogenesis, and inhibits tumor growth in mice. Thiocarbamates react with critical thiols and also complex metal ions. Using melanoma as the paradigm, we tested whether disulfiram might inhibit growth by forming mixed disulfides with critical thiols in a mechanism facilitated by metal ions. Disulfiram given to melanoma cells in combination with Cu^{2+} or Zn^{2+} decreased expression of cyclin A and reduced proliferation *in vitro* at lower concentrations than disulfiram alone. In electrophoretic mobility shift assays, disulfiram decreased transcription factor binding to the cyclic AMP-responsive element in a manner potentiated by Cu^{2+} ions and by the presence of glutathione, suggesting that thiocarbamates might disrupt transcription factor binding by inducing *S*-glutathionylation of the transcription factor DNA binding region. Disulfiram inhibited growth and angiogenesis in melanomas transplanted in severe combined immunodeficient mice, and these effects were potentiated by Zn^{2+} supplementation. The combination of oral zinc gluconate and disulfiram at currently approved doses for alcoholism also induced >50% reduction in hepatic metastases and produced clinical remission in a patient with stage IV metastatic ocular melanoma, who has continued on oral zinc gluconate and disulfiram therapy for 53 continuous months with negligible side effects. These findings present a novel strategy for treating metastatic melanoma by employing an old drug toward a new therapeutic use. [Mol Cancer Ther 2004;3(9):1049–60]

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Introduction

In the quest for effective therapies for human cancer, it is occasionally possible to apply an already approved drug toward a new use. This strategy has been most commonly used to apply cancer chemotherapeutic agents approved for one type of malignancy to the treatment of others but may also lend itself to antineoplastic application of older drugs approved for nononcologic diseases. Recently, several laboratories have investigated the aldehyde dehydrogenase inhibitor tetraethylthiuram disulfide, or disulfiram, a relatively nontoxic [oral LD_{50} of 8.6 g/kg (1)] dithiocarbamate disulfide long used for alcohol aversion therapy (2). Disulfiram reverses *in vitro* resistance of human tumors to chemotherapy drugs by blocking maturation of the P-glycoprotein membrane pump that extrudes chemotherapeutic agents from the cell (3). Disulfiram also inhibits activation of nuclear factor-κB (NF-κB) induced in human colorectal cancer cell lines by the chemotherapeutic agent 5-fluorouracil and enhances the apoptotic effect *in vitro* when the two are used in combination (4). Additionally, disulfiram inhibits DNA topoisomerases (5), induces apoptosis in cultured melanoma cells (6), reduces angiogenesis (7, 8), inhibits matrix metalloproteinases and cancer cell invasiveness (7), and retards growth of C6 glioma and Lewis lung carcinoma in mice (9). However, the mechanism for effects of disulfiram is still not clear, and the use of disulfiram is yet to be reported in the treatment of human malignancies.

The antineoplastic activity of disulfiram has been attributed to proapoptotic redox-related mitochondrial membrane permeabilization (6), zinc complexation with subsequent inhibition of Zn^{2+}-dependent matrix metalloproteinases (7), or Cu^{2+} complexation with inactivation of Cu/Zn superoxide dismutase (8, 9) and consequently diminished cellular generation of H_2O_2 from dismutation of superoxide anion (O_2^-; refs. 8, 9). Dithiocarbamates possess a RR′NC(S)SR″ functional group, giving them the ability to complex metals (10) and react with sulfhydryl groups (10) and glutathione (11). After oxidation to their corresponding disulfides, dithiocarbamates can inhibit critical sulfhydryls by forming mixed disulfides with critical cellular thiols (12), leading to such diverse effects as inhibition of caspases (12) but stimulation of mitochondrial permeability transition (13) and subsequent Bcl-independent apoptosis (14). In normal cells, the effects of other dithiocarbamates are potentiated by metals such as Cu^{2+} or Zn^{2+} (15). We therefore postulated that disulfiram might inhibit cellular proliferation of malignant tumor tumors by forming mixed disulfides, which disrupt vital protein functions, and that this process might be dependent on the presence of certain metal ions.

One potential use for this approach is treatment of malignant melanoma, a tumor notoriously resistant to radiation and traditional chemotherapeutic agents but independently sensitive *in vitro* to disulfiram (6) or metals (16). In this report, we show that disulfiram reduces activating transcription factor/cyclic AMP-responsive element binding protein (ATF/CREB) transcription factor DNA binding, cyclin A expression, cell cycle progression, and melanoma proliferation *in vitro* and in severe combined immunodeficient (SCID) mice in a manner dependent on and facilitated by copper and other heavy metal ions. In addition, we present the use of this strategy in a patient with stage IV ocular melanoma and hepatic metastases, who has experienced considerable tumor regression and remains clinically well after 53 continuous months of therapy with oral disulfiram and zinc gluconate.

Materials and Methods

Cells

Human malignant cell lines were obtained from American Type Culture Collection (Rockville, MD). Melanoma cells lines CRL1585 and CRL1619 were cultured in RPMI 1640 (Life Technologies, Grand Island, NY) with 10% fetal bovine serum (FBS) and passed with nonenzymatic cell dissociation solution (Sigma Chemical Co., St. Louis, MO). The prostate adenocarcinoma cell line CRL1435 (PC-3) and the ovarian cancer cell lines HTB75 and HTB77 were also cultured in RPMI 1640 with 10% FBS but passed with 0.05% trypsin and 0.53 mmol/L EDTA. The squamous lung carcinoma NCI-H520 and the adenosquamous lung carcinoma NCI-H596 cell lines were grown in RPMI 1640 supplemented with 10% FBS, 10 mmol/L HEPES, and 1.0 mmol/L sodium pyruvate and passed with trypsin/EDTA. All of the above were grown in a 37°C humidified environment containing 5% CO_2/air. The breast carcinoma cell line MDA-MB-453 was grown in a 37°C humidified environment with free atmospheric gas exchange, Leibovitz's L-15 medium with 2 mmol/L L-glutamine and 10% FBS, and was passed with trypsin/EDTA.

Cell Treatments

Because others have suggested that the disulfide form of dithiocarbamates is the active proximate chemical form that mediates mixed disulfide formation with protein thiols (11–13), we did most of our experiments with the tetraethylthiuram disulfide disulfiram (Sigma Chemical), which does not have a free thiol to act as an antioxidant. Malignant melanoma cells grown to confluence on 100 × 15 mm plastic Petri dishes were treated with 0 to 5 μmol/L disulfiram. These doses were chosen to approximate the steady-state plasma and tissue concentrations reported previously in humans treated with disulfiram (17). Disulfiram is converted to its bis(diethyldithiocarbamato)copper(II) complex after passage through the acid environment of the stomach (2). Therefore, Cu^{2+} was added along with disulfiram in some experiments to stimulate formation of the disulfiram-copper chelate form in which the drug is systemically absorbed. Disulfiram was dissolved in DMSO to a final concentration of <0.3% to 0.5%. Equal volumes of DMSO were added to control experiments.

The effect of disulfiram (0.15–5.0 μmol/L) or sodium diethyldithiocarbamate (1.0 μmol/L) on proliferation of malignant cell lines was studied in cultures stimulated with 10% FBS. Cell numbers were quantitated 24 to 72 hours later, as outlined below. In some experiments, disulfiram was added immediately after cells were plated. In other experiments, cells were plated and allowed to grow for 24 to 72 hours before fresh medium with disulfiram was added and cell numbers were assayed 24 to 72 hours later. Synergy was studied between disulfiram and *N*,*N*′-bis(2-chloroethyl-*N*-nitrosourea (carmustine, 1.0–1,000 μmol/L) or cisplatin (0.1–100 μg/mL) added to medium. The effect of metal ions on disulfiram was studied with 0.2 to 10 μmol/L Cu^{2+} (provided as $CuSO_4$), Zn^{2+} (as $ZnCl_2$), Ag^+ (as silver lactate), or Au^{3+} (as $HAuCl_4 \cdot 3H_2O$) ions added to growth medium, buffered to physiologic pH. To provide a biologically relevant source of copper, medium was supplemented with human ceruloplasmin at doses replicating low and high normal adult serum concentrations (250 and 500 mg/mL).

To determine whether disulfiram and metal ions might directly influence transcription factor binding, 5 μmol/L disulfiram and/or 1.6 μmol/L $CuSO_4$ (final concentrations) were added to the binding reaction of nuclear protein obtained from control cells stimulated with 10% FBS alone in the absence of drugs or metal ions. The binding reaction was done using either 2.5 mmol/L DTT or 3.0 mmol/L glutathione as the buffer reducing agent.

In additional experiments, the effect of disulfiram was studied on expression of CRE-regulated cell cycle proteins and proteins influencing apoptosis. Confluent cells were treated with 5 μmol/L disulfiram or 5 μmol/L disulfiram + 1.6 μmol/L $CuSO_4$ for 2 to 48 hours. Cells were lysed and levels of the proapoptotic protein p53, the antiapoptotic protein Bcl-2, the cyclin inhibitor $p21^{WAF1/Cip1}$, and the cyclin A and cyclin B1 were measured by immunoblots, as described below.

Potential redox effects of disulfiram were studied in three sets of experiments. The importance of cellular glutathione in thiocarbamate toxicity was studied by measuring levels of intracellular glutathione after treatment with disulfiram. Confluent monolayers were treated with disulfiram (5 μmol/L), with or without 1.6 μmol/L $CuSO_4$, and cells were harvested 24 hours later for measurement of glutathione. To assess whether a pro-oxidant effect of disulfiram accounts for growth inhibition, we studied the effect of the potent lipophilic antioxidant probucol (1.0–1,000 μmol/L) on the antiproliferative effect of disulfiram. Finally, generation of intracellular oxidants in response to disulfiram (0.625–5 μmol/L), Cu^{2+} (0.2–1.6 μmol/L $CuSO_4$), or 1.25 μmol/L disulfiram + various concentrations of Cu^{2+} was measured directly, as outlined below.

Dithiocarbamates have been reported to inhibit proliferation of malignant cells by reducing cyclooxygenase-2 production of mitogenic prostaglandins (18). To explore the role of cyclooxygenase inhibition on tumor growth, cells were cultured with or without disulfiram in the presence or absence of the cyclooxygenase-1 and cyclooxygenase-2 inhibitors indomethacin (5 μg/mL) or sodium salicylate (1 mmol/L). Dithiocarbamates have also been shown to increase cytoplasmic levels of nitric oxide ($NO^{\bullet}$) by decomposing *S*-nitrosoglutathione (19). $NO^{\bullet}$ could in turn induce mitochondrial permeability transition and apoptosis. To probe whether disulfiram might be inducing growth retardation by altering $NO^{\bullet}$ production, proliferation was studied with and without disulfiram in the presence and absence of the $NO^{\bullet}$ synthase inhibitor N^{ω}-nitro-L-arginine added to growth medium (100 μmol/L).

Finally, several dithiocarbamate effects have been attributed to increasing the intracellular levels of cupric ions (15, 20). To further probe the role of cupric ions in mediating cytotoxicity from disulfiram, cells were cultured with or without addition of the impermeate Cu^{2+} chelator bathocuproinedisulfonic acid (BCPS, 50 or 100 μmol/L) added to medium to sequester Cu^{2+} in the extracellular compartment. Cells were also treated for 12 hours with various concentrations of disulfiram (0.625–5.0 μmol/L) and intracellular copper levels were measured as outlined below.

Electrophoretic Mobility Shift Assays

Nuclear protein was isolated and DNA binding reactions were done and quantitated as detailed previously (21) using consensus oligonucleotides 5′-AGAGATTGCCTGACGTCAGAGAGCTAG-3′ and 3′-TCTCTAACGGACTGCAGTCTCTCGATC-5′ for the CRE and 5′-AGTTGAGGGGACTTTCCCAGGC-3′ and 3′-TCAACTCCCCTGAAAGGGTCCG-5′ for NF-κB (p50; Promega, Madison, WI). Competition experiments were done with 10× unlabeled wild-type oligonucleotide sequences for CRE or NF-κB. Supershift experiments were done by incubating the binding reaction with 1 μg supershifting antibody (Santa Cruz Biotechnology, Santa Cruz, CA) prior to electrophoresis.

Measurement of Proliferation in Cell Cultures

Proliferation of cultured cells seeded into 24-well uncoated plastic plates (Costar, Corning, NY) at 50,000 cells per well was quantitated as detailed previously (22) using a colorimetric method based on metabolic reduction of the soluble yellow tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble purple formazan by the action of mitochondrial succinyl dehydrogenase. This assay was confirmed by experiments in which cells were stained with Wright's modified Giemsa, counterstained with eosin, and counted directly at a magnification of ×100 using a 1 mm^2 ocular grid.

Measurement of Apoptosis

Apoptosis was studied by terminal deoxynucleotidyl transferase–dependent 3′-OH fluorescein end labeling of DNA fragments using a Fluorescein-FragEL DNA fragmentation detection kit (Oncogene Research Products, Cambridge, MA), by fluorescent-labeled Annexin V staining of phosphatidylserine translocated to the membrane surface using the Annexin V-FLUOS staining kit (Roche Molecular Biochemical, Indianapolis, IN), and by visually assessing endonuclease-dependent DNA fragmentation on ethidium bromide–stained agarose gels.

DNA Cell Cycle Measurements

To study the effect of disulfiram on the DNA cell cycle, confluent cells were treated with 10% FBS + DMSO vehicle, 10% FBS and DMSO vehicle + 250 mg/mL ceruloplasmin as a source of Cu^{2+}, 10% FBS + 5 μmol/L disulfiram, or 10% FBS + 5 μmol/L disulfiram and 250 mg/mL ceruloplasmin. After 24 hours, cells were trypsinized, washed twice in cold Dulbecco's PBS with 1 mmol/L EDTA and 1% bovine serum albumin, fixed for 30 minutes in ice-cold 70% ethanol, and stained by incubation for 30 minutes at 37°C in a 10 mg/mL solution of propidium iodide in Dulbecco's PBS and 1 mg/mL RNase A. DNA cell cycle measurements were made using a FACStarPlus flow cytometer (Becton Dickinson, San Jose, CA).

Immunoblots for Proteins

Immunoblots were done and quantitated as described previously (22) using primary rabbit polyclonal antibodies against human Bcl-2, p53, $p21^{WAF1/Cip1}$, cyclin A and cyclin B1, and peroxidase-labeled donkey polyclonal anti-rabbit IgG (Santa Cruz Biotechnology).

Measurement of Intracellular Copper

Cells were cultured in 12-well plastic tissue culture plates at an initial plating density of 50,000 cells per well, grown to confluence, and treated with disulfiram or DMSO vehicle, as outlined above. Medium was removed and cells were washed twice with Dulbecco's PBS. Cells were scraped into 1.0 mL of 3 N HCl/10.0% trichloroacetic acid and hydrolyzed at 70°C for 16 hours. The hydrolysate was centrifuged at 600 × *g* for 10 minutes to remove debris and copper was measured in the supernatant using inductively coupled plasma emission spectroscopy (model P30, Perkin-Elmer, Norwalk, CT) at wavelengths of 325.754 and 224.700 nm. To minimize metal contamination, plastic ware rather than glassware was used in these experiments, and double-distilled deionized water was used for all aqueous medium. Results are reported as nanograms of copper per culture well.

Measurement of Intracellular Generation of Reactive Oxygen Species

Generation of reactive oxygen species in response to disulfiram with or without $CuSO_4$ was studied using 2′7′-dichlorofluorescin diacetate (Molecular Probes, Eugene, OR) and a modification of methods reported previously (23). Cells were plated in 24-well plastic plates at 50,000 cells per well and grown to confluence. Medium was aspirated from wells and replaced with 100 μL medium containing 10 μmol/L 2′7′-dichlorofluorescin diacetate, and plates were incubated at 37°C for 30 minutes. The 2′7′-dichlorofluorescin diacetate containing medium was aspirated, cells were washed twice with medium alone, and fresh medium (100 μL) was added to wells. With the plate on the fluorescence microplate reader (HTS 7000), cells were stimulated with 25 μL medium containing 5× concentrations of disulfiram and/or $CuSO_4$ to provide final concentrations of 0 to 5.0 μmol/L disulfiram and/or 0 to 1.6 μmol/L $CuSO_4$, respectively. The relative concentration of dichlorofluorescin was measured immediately by monitoring fluorescence at 37°C using an excitation wavelength of 485 nm and emission wavelength of 535 nm.

Measurement of Intracellular Glutathione

Disulfiram (5 μmol/L), with or without 1.6 μmol/L $CuSO_4$, was added to cells grown to confluence on 100 × 15 mm plastic dishes, and cells were harvested 24 hours later for measurement of glutathione using the 5,5′-dithiobis(2-nitrobenzoic acid)-glutathione reductase recycling assay (24).

Synthesis of Thiocarbamate-Metal Chelates

Synthesis of diethyldithiocarbamato-metal complexes is known in the literature. Typically, aqueous solutions of a metal ion (e.g., $CuCl_2$) and sodium or ammonium diethyldithiocarbamate are mixed and the desired complex was separated by extraction into an organic phase such as dichloromethane. The stoichiometric ratio between metal ion and diethyldithiocarbamate salt can influence the final stoichiometry of the product. Identical complexes were synthesized starting with disulfiram rather than diethyldithiocarbamate. All diethyldithiocarbamato-metal complexes were characterized by a single crystal X-ray diffraction and structures were reported in the Cambridge Crystallographic Database. The Ag^+ and Zn^{2+} diethyldithiocarbamate complexes were found to be polymeric. As an example, the results for the Au^{3+} complex are detailed.

Study of Antitumor Activity of Disulfiram and Zinc Supplementation *In vivo*

Adult female CB17-SCID mice (Harlan, Indianapolis, IN) were housed in a protected laminar flow facility with access to water and either a standard diet containing 87 ppm zinc or a zinc-supplemented diet (Harlan) containing 1,000 ppm Zn^{2+} as zinc acetate. Mice were injected s.c. in the right groin with 5×10^6 cells from a highly aggressive malignant melanoma obtained from a Carolinas Medical Center patient. The frozen tumor was passaged twice in SCID mice to adapt it to *in vivo* growth before use in these experiments. On the day of tumor injection, all mice began daily administration of drug. Drug was given in a total volume of 0.2 mL by gastric gavage via smooth Teflon-tipped needles inserted transorally into the stomach. Four groups were studied: tumor control (n = 10; 0.2 mL olive oil daily; zinc diet of 87 ppm); zinc-supplemented control (n = 10; 0.2 mL olive oil daily; zinc diet of 1,000 ppm); disulfiram (n = 10; 200 mg/kg/d disulfiram in 0.2 mL olive oil; zinc diet of 87 ppm); and zinc-supplemented diet + disulfiram (n = 10; 200 mg/kg/d disulfiram in 0.2 mL olive oil; zinc diet of 1,000 ppm). Mice were examined daily, the tumor was measured in two dimensions, and the tumor volume was estimated using the formula for an elipse. When estimated tumor volume approached 500 mm^3 within any animal, all mice were euthanized. This protocol was reviewed and approved by the Institutional Animal Care and Use Committee at Carolinas Medical Center. Tumors were excised, weighed, fixed in formalin, sectioned, and stained with H&E or immunostained for factor VIII. Slides were coded and examined by a blinded observer who identified vessels as deposits of red cells. For each slide, the number of vessels was counted in four different fields representative of the tumor. The average number of vessels per field was averaged per biopsy specimen and used to evaluate tumor vascularity.

Results

Disulfiram Inhibits Melanoma Proliferation in a Metal-Dependent Fashion

In concentrations reported in humans (17), disulfiram inhibited melanoma proliferation *in vitro* in a dose-dependent fashion, with near complete growth inhibition at 5 μmol/L ($P < 0.001$; Fig. 1), and increased the number of apoptotic cells in culture (Fig. 2). Within the same concentration ranges, disulfiram likewise inhibited growth of other malignant cells (IC_{50}: 2.5 μmol/L for CRL1585 melanoma, 2.5 μmol/L for PC-3 prostate adenocarcinoma; 0.625 μmol/L for H520 squamous cell lung cancer,

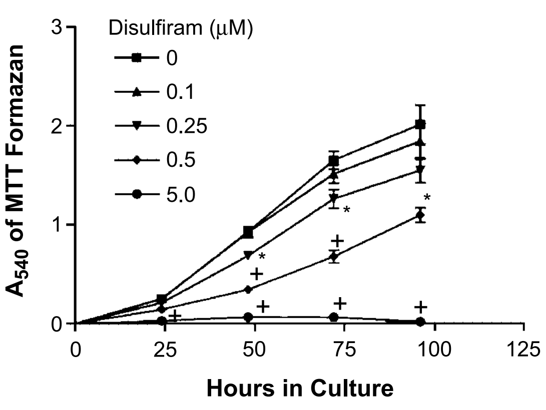

Figure 1. Disulfiram inhibits proliferation of CRL1619 human melanoma cells. Cells stimulated with 10% FBS were plated at a density of 50,000 cells per well, and DMSO vehicle (5 μL/mL) or disulfiram was added to wells at the indicated concentrations. After 24, 48, 72, or 96 hours, proliferation was quantitated by assessing the cell number–dependent reduction of the soluble yellow tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble formazan, measured as the absorbance at 540 nm (A_{540}). Two-way ANOVA shows $P < 0.001$ for group, time, and group-time interaction. *, $P < 0.01$, at similar culture time versus DMSO vehicle. +, $P < 0.001$, at similar culture time point versus DMSO vehicle.

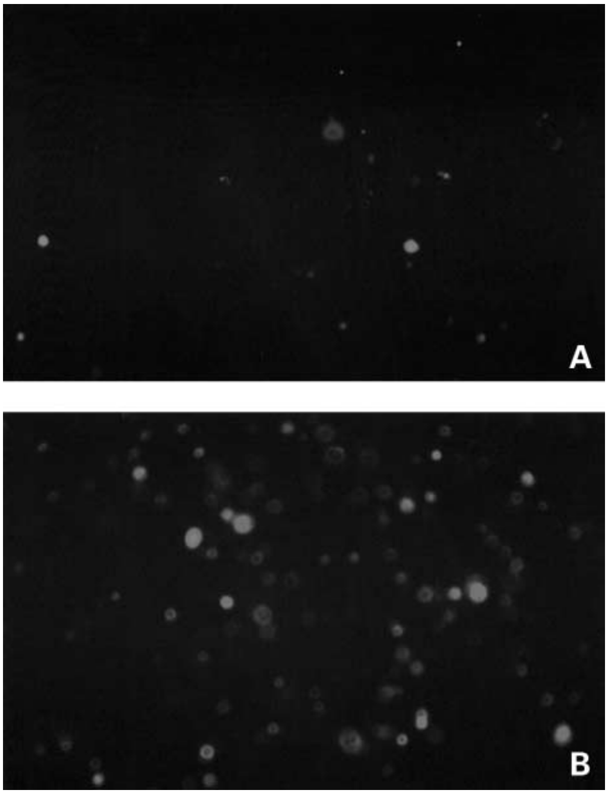

Figure 2. Disulfiram induces apoptosis in melanoma measured by 3′-OH fluorescein end labeling of DNA fragments. **A,** CRL1619 melanoma cells treated with DMSO vehicle and 3′-OH fluorescein end labeled. **B,** CRL1619 melanoma cells treated with disulfiram (5 μmol/L) and 3′-OH fluorescein end labeled. Cells were grown to confluence on 35 mm Petri dishes or on glass slides and treated for 12 hours with disulfiram or DMSO as vehicle. Apoptosis was studied by terminal deoxynucleotidyl transferase–dependent 3′-OH fluorescein end labeling of DNA fragments using a Fluorescein-FragEL DNA fragmentation detection kit.

1.25 μmol/L for H596 adenosquamous cell lung cancer, and 0.625 μmol/L for MDA-MB-453 breast carcinoma). Disulfiram also augmented the antiproliferative effect of cisplatin or carmustine on melanoma cells (4 ± 1% inhibition of growth at 24 hours with 100 ng/mL cisplatin alone versus 17 ± 3% inhibition with cisplatin and 2.5 μmol/L disulfiram; $P < 0.05$; 46 ± 7% stimulation of growth at 24 hours with 10 μmol/L carmustine alone versus 75 ± 6% inhibition of growth with carmustine and 0.6 μmol/L disulfiram; $P < 0.001$), suggesting that it might reduce resistance to chemotherapy, as reported recently (3, 4).

Because thiocarbamates chelate metals (10), we explored whether growth inhibition was contingent on the ability of disulfiram to complex with metal ions from growth medium. Disulfiram increased intracellular copper in melanoma monolayers (ng copper per well: 56 ± 7 for control, 52 ± 4 for DMSO vehicle, 102 ± 5 for 1.25 μmol/L disulfiram, 160 ± 17 for 2.5 μmol/L disulfiram, 195 ± 3 for 5.0 μmol/L disulfiram; all $P < 0.01$ versus control or vehicle). Adding the cell impermeate Cu^{2+} chelator BCPS to growth medium reversed the antiproliferative activity

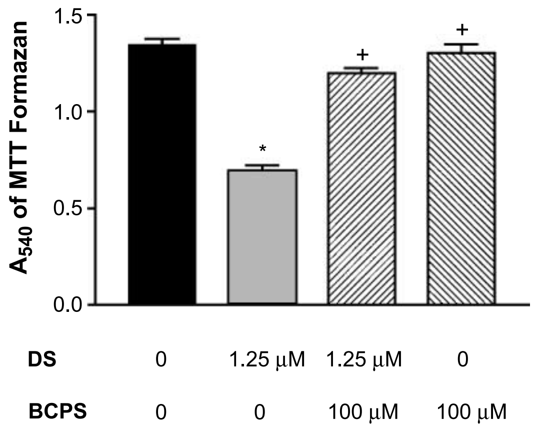

Figure 3. Complexation of Cu^{2+} reduces the antiproliferative activity of disulfiram. CRL1619 human melanoma cells stimulated with 10% FBS were plated at a density of 50,000 cells per well and treated with the indicated concentrations of DMSO vehicle (5 μL/mL) or disulfiram (*DS*, 1.25 μmol/L) with or without the cell impermeate Cu^{2+} chelator *BCPS* to complex Cu^{2+} and trap it in the extracellular medium. BCPS reversed growth the antiproliferative activity of disulfiram in a dose-dependent manner. % Growth inhibition at 48 hours: 48 ± 2% with disulfiram (1.25 μmol/L); 11 ± 2% with disulfiram + BCPS (100 μmol/L); 3 ± 3% with BCPS alone (100 μmol/L). *, $P < 0.001$ versus untreated. +, $P < 0.001$ versus disulfiram.

of disulfiram (Fig. 3). Conversely, growth inhibition was enhanced by supplementing medium with cupric ion concentrations that do not by themselves affect cell growth (Fig. 4). Ovarian and lung cancer cell lines exhibited similar reversal of disulfiram-induced growth inhibition with

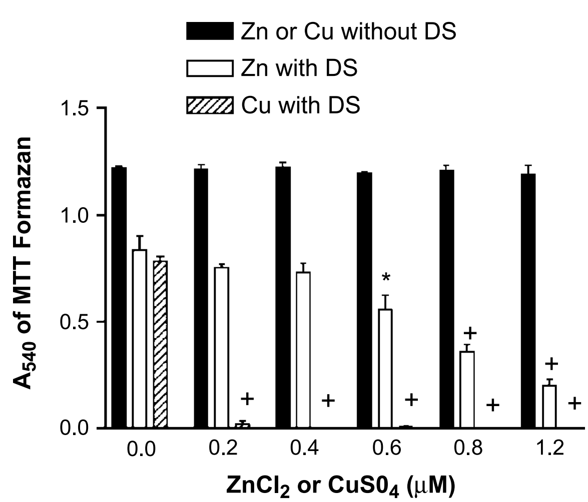

Figure 4. Supplementation of growth medium with Cu^{2+} or Zn^{2+} enhances the antiproliferative activity of disulfiram. CRL1619 human melanoma cells stimulated with 10% FBS were plated at a density of 50,000 cells per well and treated with the indicated concentrations of DMSO vehicle (5 μL/mL) or disulfiram (*DS*, 0.625 μmol/L) and concentrations of $CuSO_4$, $ZnCl_2$, or metal ions + DMSO or disulfiram. After another 24 hours, proliferation was quantitated as in Fig. 1. Addition of $CuSO_4$ (0.2 μmol/L) to medium converts disulfiram (0.625 μmol/L) from IC_{50} to IC_{100} of drug. *, $P < 0.01$ and +, $P < 0.001$ compared with no $CuSO_4$ or $ZnCl_2$.

Table 1. Effect of complexation or supplementation of cupric ions on antiproliferative activity of disulfiram

Treatment	% Growth inhibition	
	HTB75 Ovarian Cancer	HTB77 Ovarian Cancer
Disulfiram (0.5 μmol/L)	75 ± 4	81 ± 2
Disulfiram (0.5 μmol/L) + BCPS (200 μmol/L)	0 ± 4*	13 ± 5*
Disulfiram (0.1 μmol/L)	12 ± 4	5 ± 2
Disulfiram (0.1 μmol/L) + $CuSO_4$ (0.8 μmol/L)	75 ± 2*	83 ± 1*
	520 Squamous Lung Cancer	596 Adenosquamous Lung Cancer
Disulfiram (0.5 μmol/L)	76 ± 3	69 ± 2
Disulfiram (0.5 μmol/L) + BCPS (200 μmol/L)	0 ± 2*	5 ± 6*
Disulfiram (0.25 μmol/L)	66 ± 2	53 ± 4
Disulfiram (0.25 μmol/L) + $CuSO_4$ (0.8 μmol/L)	88 ± 2†	91 ± 1*

NOTE: Cells stimulated with 10% FBS were plated at a density of 50,000 cells per well, and DMSO vehicle (5 μL/mL) or disulfiram was added to wells at the indicated concentrations. To decrease the concentration of available Cu^{2+}, the impermeate Cu^{2+} chelator BCPS was added to medium. To increase the available Cu^{2+}, medium was supplemented with $CuSO_4$. After 48 hours, proliferation was quantitated by assessing the cell number–dependent reduction of the soluble yellow tetrazolium dye 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble formazan, measured as the absorbance at 540 nm.
*$P < 0.001$ versus respective disulfiram concentration alone.
†$P < 0.01$ versus respective disulfiram concentration alone.

BCPS and enhancement of disulfiram-induced growth inhibition by cupric ions (Table 1). *In vivo*, one potential source of cupric ions is the copper transport protein ceruloplasmin, which has complexable cupric ions (25) that could serve as a source of copper to enhance disulfiram. Although ceruloplasmin alone has no effect (0 ± 0% growth inhibition with 250 mg/mL human ceruloplasmin), the addition of ceruloplasmin to disulfiram significantly enhances dithiocarbamate-induced growth inhibition (70 ± 2% growth inhibition at 24 hours with 0.625 μmol/L disulfiram; 100 ± 0% growth inhibition with disulfiram + ceruloplasmin; $P < 0.001$). Disulfiram treatment of melanoma cells (Fig. 5) slightly reduces the number of cells in G_0-G_1 and increases the portion in S phase of the cell cycle. Ceruloplasmin greatly magnifies these effects and produces S-phase cell cycle arrest. Thus, the antiproliferative effect of disulfiram seems codependent on Cu^{2+}. Taken together, these results suggest that the inhibitory effect of disulfiram is critically dependent on the binding of cupric ions from the extracellular medium and transporting them as a thiocarbamate-metal complex into cells.

Treatments that increase intracellular Cu^{2+} might be expected to enhance generation of reactive oxygen species. However, disulfiram did not deplete glutathione (nmol glutathione/μg cell protein: 228 ± 18 in untreated cells, 254 ± 7 in DMSO vehicle controls, and 273 ± 11 in cells with 5 μmol/L disulfiram), and the combination of 5.0 μmol/L disulfiram and 1.6 μmol/L $CuSO_4$ even increased glutathione (293 ± 16 nmol glutathione/μg cell protein; $P < 0.05$ compared with untreated cells). Likewise, neither disulfiram (0.625–5 μmol/L), $CuSO_4$ (0.2–1.6 μmol/L), or the combination of 1.25 μmol/L disulfiram and 0.2 to 1.6 μmol/L $CuSO_4$ caused oxidation of dichlorofluro rescin. The baseline fluorescence of 1,431 ± 23 units was not increased by any of the treatments. In addition, the

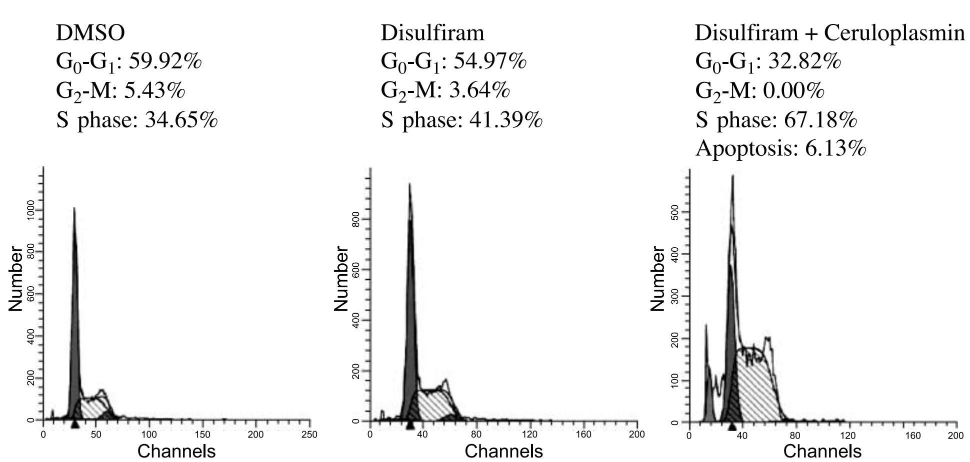

Figure 5. Disulfiram combined with Cu^{2+} induces S-phase cell cycle arrest in CRL1619 melanoma cells and apoptosis. Unsynchronized CRL1619 melanoma cells were grown in the presence of DMSO vehicle, disulfiram (5 μmol/L), or disulfiram (5 μmol/L) + ceruloplasmin (250 mg/mL) as a source of Cu^{2+}. After 24 hours, cells were harvested and flow cytometric cell cycle analysis was done. The proportion of nuclei in each phase of the cell cycle was determined with ModFit DNA analysis software. Cells in G_0-G_1 and G_2-M are in *red*, cells in S phase are *hatched*, and apoptotic cells are in *blue*. Disulfiram increases the portion of cells in S phase. The combination of disulfiram and ceruloplasmin further increases the number of cells in S phase, prevents progression into the G_2-M cell cycle, and induces apoptosis. Six percent of cells are apoptotic, over two thirds of cells are in S phase, and none are in G_2-M.

antioxidant probucol did not prevent disulfiram from reducing melanoma proliferation. Augmentation of intracellular copper might also increase levels of $NO^{\bullet}$ through Cu^{2+}-mediated decomposition of nitrosothiols (19). $NO^{\bullet}$ might in turn induce mitochondrial permeability transition and apoptosis (26). However, although the $NO^{\bullet}$ synthase inhibitor *N*-nitro-L-arginine alone slightly enhanced cellular growth, it did not eliminate the antiproliferative effect of disulfiram (data not shown). Thus, disulfiram does not affect cellular redox state. Finally, other thiocarbamates have been postulated to interfere with growth of colorectal carcinoma by reducing expression of cyclooxygenase-2 (18). However, cyclooxygenase inhibitors failed to reduce melanoma growth (data not shown).

NF-κB inhibition by thiocarbamates has been associated recently with facilitation of intracellular zinc transport (27), and zinc supplementation increases the toxicity of thiocarbamates for vascular smooth muscle cells (15). Zinc substantially enhanced the antiproliferative potential of disulfiram against melanoma cells (Fig. 4). Dithiocarbamates can also chelate other metals (28), and gold and silver salts also enhanced the antiproliferative activity of disulfiram (growth inhibition: 45 ± 5% with 0.15 μmol/L disulfiram; 0 ± 0% with 5 μmol/L silver lactate alone; 71 ± 7% with disulfiram + silver lactate, $P < 0.001$; 0 ± 0% with 5 μmol/L gold tetrachloride alone; 99 ± 1% with disulfiram + gold tetrachloride, $P < 0.001$). In light of these findings, we synthesized chelates of disulfiram with Au^{3+}, Cu^{2+}, Zn^{2+}, Ag^{+}, Ga^{3+}, or Fe^{3+}. X-ray crystallography confirmed the structures as diethylthiocarbamato complexes of respective metal ions (complexes with Au^{3+} shown in Fig. 6; others are in online supplemental data). To confirm that the proximate reactive dithiocarbamate structure important for promoting cellular mixed disulfide formation is the thiolate anion generated from fully reduced dithiocarbamates by metals, we compared the antiproliferative activity of the thiolate sodium diethyldithiocarbamate alone or in the presence of a low concentration of DTT to promote formation of the fully reduced thioacid. Sodium diethyldithiocarbamate alone (1 μmol/L) decreased melanoma proliferation by 92 ± 2% after 48 hours ($P < 0.001$), but growth was inhibited by only 24 ± 3% ($P < 0.001$) with simultaneous addition of a concentration of DTT (100 μmol/L), which does not affect proliferation of melanoma cells by itself (0 ± 0 %). Thus, the function of metals may be to facilitate formation of the dithiocarbamate anion, which might condense into mixed disulfides with critical protein sulfhydryls (11–13).

Disulfiram and Metals Inhibit ATF/CREB DNA Binding and Cyclin A Expression

One critical location of cysteines is the DNA binding region of transcription factors, wherein sulfhydryls generally must remain reduced to insure effective transcription factor binding (29). When cysteines in the positively charged transcription factor DNA binding domain are oxidatively modified, repair processes are triggered that result in formation of mixed disulfides between glutathione and protein thiols (29, 30). Consequent to protein *S*-glutathionylation, the usually positively charged transcription factor DNA binding domain develops a negative charge imparted by the dual carboxylate end groups of glutathione, thereby repelling similarly charged DNA and disrupting DNA transcription factor binding (29). The transcription factors NF-κB, activator protein-1, and ATF/CREB all contain cysteines in their DNA binding regions as reactive sites for mixed disulfide formation (29, 31–35). To determine if thiocarbamates might form mixed disulfides with these sulfhydryls, we studied DNA binding of the CRE, which is of pivotal importance for melanoma proliferation (36–38). Melanomas exhibited prominent constitutive DNA binding activity for CRE (Fig. 7A) that was significantly reduced by treatment of cells with disulfiram and Cu^{2+} (Fig. 7B). Disulfiram and Cu^{2+} also inhibited DNA binding of NF-κB (data not shown). To determine if inhibition was from direct transcription factor modification, we added each agent directly to the binding reaction (Fig. 7C). Cu^{2+} facilitated inhibition of CRE DNA binding by disulfiram (lane 5), suggesting that metal ions might enhance formation of a mixed disulfide between the thiuram disulfide and cysteine sulfhydryls in the transcription factor DNA binding region. Synergistic inhibition of transcription factor DNA binding by Cu^{2+} and disulfiram was even more pronounced when DTT was replaced by glutathione as the reducing agent in the binding buffer (lane 9). This suggests that glutathione, found in millimolar concentrations within the nucleus (30), might react with the mixed disulfide

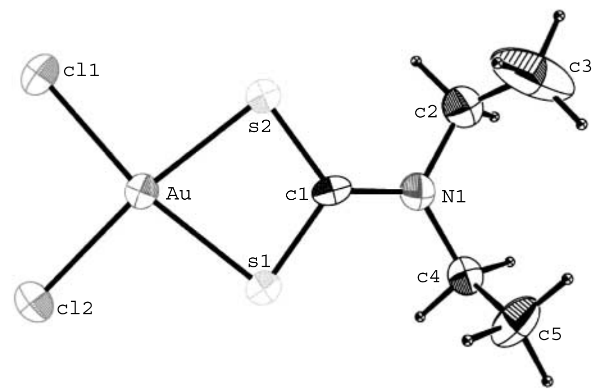

Figure 6. X-ray crystallographic structure of Au^{3+} diethyldithiocarbamate. Complexes were generated as outlined in Materials and Methods. A Nonius Kappa-CCD diffractometer was used to collect X-ray diffraction data. The crystal diffracted well and a data set was collected to 27.5° in θ using Mo *K*α radiation (λ = 0.71073 Å). Least squares refinement on the cell variables revealed an orthorhombic unit cell with a = 11.5167(5), b = 7.2472(2), c = 12.9350(7) Å, and a volume of 1,079.6(1) $Å^3$. Examination of the systematic absences showed the space group to be Pnma (#62). The structure was solved by direct methods using SIR92 and revealed the crystal to be dichloro(diethylthiocarbamato)gold(III). The structure was confirmed by the successful solution and refinement of the 83 independent variables for the 893 reflections [$I > 3\delta(I)$] to *R* factors of 3.3% and 3.2%, with an ESD of 1.499. The gold complex is a square planar coordination complex in which the *Au* and the four coordinated atoms sit on a mirror at x, 0.25, z. The organic ligand was found to be disordered with the diethylamine ligand occupying two sites related to each other through the mirror plane. This compound inhibited CRL1619 melanoma growth by 81 ± 1% after exposure for 48 hours to a concentration as low as 0.25 μmol/L.

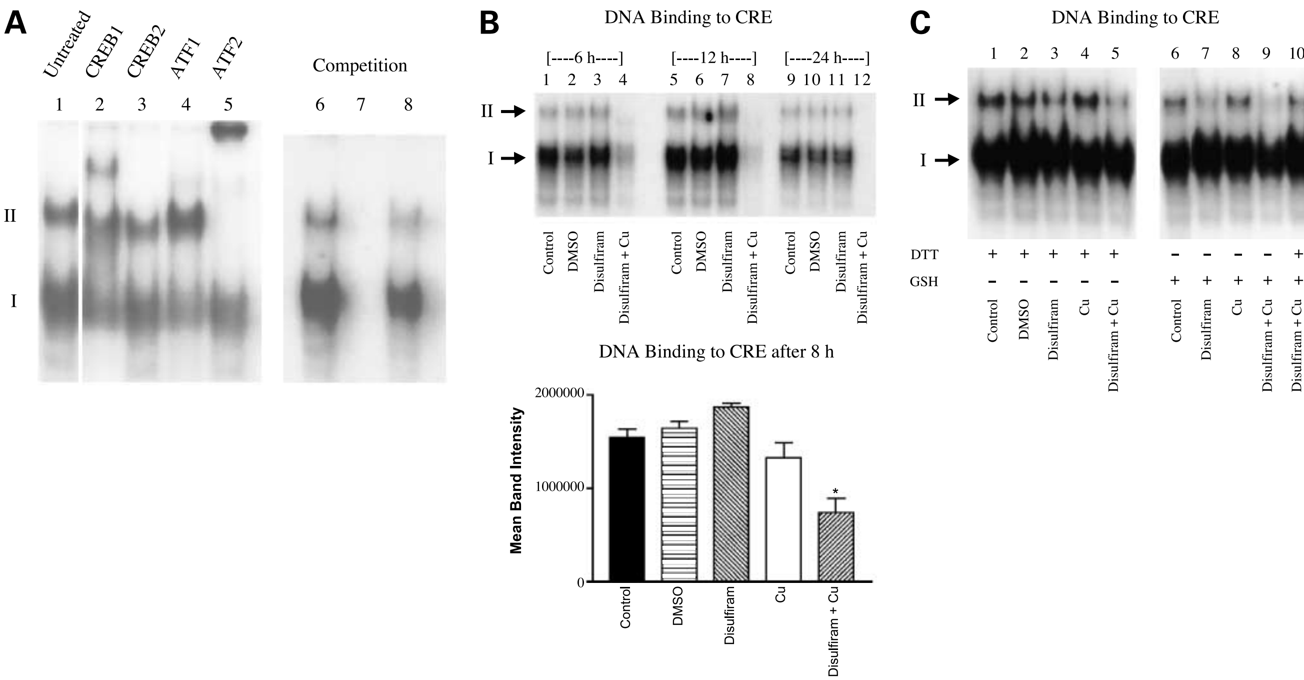

Figure 7. Disulfiram and metals inhibit transcription factor binding to the CRE. **A,** CRL1619 melanoma cells exhibit constitutive DNA binding activity to the CRE (*lane 1*). CRL1619 melanoma cells were grown to 60% confluence on 100 × 15 mm plastic Petri dishes, nuclear protein was harvested, and electrophoretic mobility shift assays were done using the consensus oligonucleotides 5′-AGAGATTGCCTGACGTCAGAGAGCTAG-3′ and 3′-TCTCTAACGGACTGCAGTCTCTCGATC-5′ for the CRE, end labeled by phosphorylation with [γ-^{32}P]ATP and T4 polynucleotide kinase. CRE complexes (*I* and *II*) are labeled. Supershift experiments done by incubating the binding reaction with antibody (1 μg) before addition of labeled probe show that *complex II* contains ATF2 (*lane 5*), whereas *complex I* is composed primarily of CREB1 (*lane 2*), with some *ATF1* (*lane 4*). Competition experiments in *lanes 6–8* show specificity of the DNA binding reaction: untreated (*lane 6*); with 10× unlabeled CRE probe added to binding reaction (*lane 7*); with 10× unlabeled NF-κB probe added to binding reaction (*lane 8*). **B,** treatment of melanoma cells with disulfiram and Cu^{2+} inhibits transcription factor binding to CRE. CRL1619 melanoma cells were grown to 80% confluence, nuclear protein was harvested, and electrophoretic mobility shift assays were done for the CRE. *Top,* treatment of cultures for 6, 12, or 24 hours with the combination of disulfiram (5 μmol/L) and $CuSO_4$ (*Cu*, 1.6 μmol/L) substantially interrupted transcription factor binding to CRE. The ATF2 containing *complex II* has proven to be the more sensitive to inhibition. *Bottom,* electrophoretic mobility shift assays were done using nuclear protein from replicate experiments ($n = 4$) in which near confluent cells were treated for 8 hours and densitometry was done on the ATF2 containing *complex II*. The combination of disulfiram + Cu^{2+} reduced DNA binding by half. *, $P < 0.05$ compared with other treatments. **C,** the inhibitory effects of disulfiram or disulfiram + Cu^{2+} on transcription factor binding are potentiated in the presence of glutathione (*GSH*). Electrophoretic mobility shift assays were done with addition of disulfiram or disulfiram + $CuSO_4$ (*Cu*, 1.6 μmol/L) directly to the binding reaction of nuclear protein and oligonucleotides. Disulfiram alone reduced DNA binding to CRE in the upper ATF2 containing *complex II* (*lane 3*). This was magnified when disulfiram was combined with Cu^{2+} ions (*lane 5*). Results are consistent with modest disruption of ATF2 binding to CRE from formation of mixed disulfides between disulfiram and cysteines in the DNA binding region and greater disruption when Cu^{2+} is present to enhance mixed disulfide formation. However, reduction in CRE binding was much more pronounced when the binding reaction was done with *GSH* instead of *DTT* as the reducing agent (*lane 7* for disulfiram, *lane 9* for disulfiram + Cu^{2+}). Inhibition of ATF2 containing *complex II* binding to CRE by disulfiram and Cu^{2+} in the presence of *GSH* was reversed by simultaneous addition of the potent uncharged reducing agent *DTT* (*lane 10*).

formed between the dithiocarbamate and protein cysteine sulfhydryls (11), leading to a bulky, negatively charged glutathione-containing mixed disulfide that can more effectively disrupt DNA binding. Disulfiram and Cu^{2+} also reduced expression of cyclin A (Fig. 8), which is positively regulated by a CRE element (39), a phenomenon that would be expected to reduce cell cycle progression into G_2-M (Fig. 5). Disulfiram had no consistent effect on expression of cyclin B1, p21$^{WAF1/Cip1}$, p53, or Bcl-2.

Disulfiram and Zn^{2+} Inhibit Melanoma Growth and Angiogenesis in Mice

Melanoma cells transplanted into SCID mice grew rapidly as a spherical encapsulated mass. Tumor volume reached ~500 mm^3 in controls by 16 days, when animals were sacrificed. Zn^{2+} alone had no affect on tumor growth (Fig. 9). However, treatment with disulfiram alone or disulfiram + Zn^{2+} significantly inhibited tumor growth. In mice receiving disulfiram and a Zn^{2+}-enriched diet, tumors were less than one third (83 ± 12 mg) of the size of tumors in either controls (289 ± 57 mg) or mice receiving a zinc-enriched diet alone (271 ± 19 mg). Histologic sections of tumors from mice treated with disulfiram + zinc showed more cellular necrosis. There was also a significant reduction in the number of blood vessels per field in disulfiram-treated or disulfiram + zinc acetate–treated mice, suggesting that thiocarbamates inhibit angiogenesis (vessels per field: 5.8 ± 0.8 for control; 5.4 ± 1.6 for zinc supplemented; 2.5 ± 0.7 for disulfiram, $P < 0.05$ versus control; 2.0 ± 0.7 for disulfiram + zinc, $P < 0.05$ versus control). Mice in all groups tolerated treatment well, although diarrhea was noted in animals receiving disulfiram + Zn^{2+}-enriched diet.

Case Report: Use of Disulfiram and Zn^{2+} for Treatment of Metastatic Melanoma in a Patient

We also report the first use of disulfiram and Zn^{2+} to treat advanced stage IV metastatic melanoma in a patient. This was done with approval from the Carolinas Medical Center Institutional Review Board, informed consent was obtained, data were collected prospectively, and the patient has been on no other treatment for melanoma. The subject treated is a 64-year-old woman who presented with a nonoperable central liver metastasis from a T2 ocular melanoma that had been removed 5 years previously. She had developed abdominal pain and was found to have a 2.3 cm right hepatic metastasis and a 5.5 cm central liver metastasis confirmed as recurrent melanoma by biopsy. She declined chemotherapy, interleukin-2 therapy, or liver perfusion. After granting informed consent, she was started on 250 mg/d disulfiram (Antabuse, Wyeth, Madison, NJ) with the largest meal of the day. This dose was increased to 500 mg/d after 1 month. Zinc gluconate (50 mg chelated elemental Zn^{2+}, General Nutrition Center, San Francisco, CA) was also given thrice daily but not concurrent with disulfiram administration. This heavy metal and its dose were chosen for previously demonstrated safety in humans as the preventative treatment for Wilson's disease. Doses of each agent were those currently recommended for treatment of alcoholism and Wilson's disease, respectively. On starting the protocol, the patient suffered grade 1 (National Cancer Institute Common Toxicity Criteria, version 2.0) diarrhea, nausea, depression, and malaise. Except for nausea, these side effects resolved within 2 months of continued treatment. Her abdominal pain also completely resolved and she returned to work. After 9 months, disulfiram was reduced to 250 mg/d and her nausea ceased. She has continued on disulfiram 250 mg/d and zinc gluconate 50 mg thrice daily. All laboratory studies have remained normal. Repeat computed tomography and positron emission tomography scans after 3 months of therapy showed a >50% reduction in tumor size (Fig. 10, *top*). A positron emission tomography scan 12 months after initiating treatment showed the lesions to be stable (Fig. 10, *bottom*), and the most recent computed tomography scan after 42 months of treatment (Fig. 10, *top, far right*) shows that residual hepatic disease has remained stable. She continues to be clinically well and physically active after 53 continuous months of therapy.

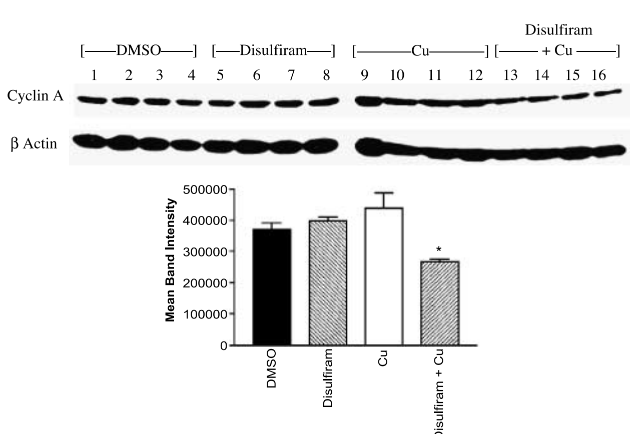

Figure 8. Disulfiram and Cu^{2+} reduce expression of the cell cycle protein cyclin A. Although disulfiram or Cu^{2+} alone had little effect, treatment with the combination of disulfiram + Cu^{2+} reduced expression of cyclin A by 24 hours, which would be expected to produce a site of cell cycle arrest consistent with that seen in Fig. 3. CRL1619 melanoma cells were plated at equal densities, grown to 80% confluence, and, in replicate experiments (n = 4 each), treated with DMSO vehicle (*lanes 1–4*), disulfiram (5 μmol/L, *lanes 5–8*), $CuSO_4$ (*Cu*, 1.6 μmol/L, *lanes 9–12*), or the combination of disulfiram and $CuSO_4$ (*lanes 13–16*). After 24 hours, immunoblots were done to assay for cyclin A. *Bottom,* quantitation of experiments by densitometry. *, $P < 0.05$ compared with all other treatments.

Discussion

In this report, we show that disulfiram reduces cyclin A expression, cell cycle progression into G_2-M, and melanoma proliferation *in vitro* in a manner both dependent on and facilitated by heavy metal ions. In the presence of heavy metal ions, disulfiram also substantially inhibits growth of human melanomas in SCID mice and reduces angiogenesis in the implanted tumors. When disulfiram and zinc gluconate were coadministered to a patient with stage IV metastatic ocular melanoma, the subject experienced impressive resolution of hepatic metastases with minimal side effects. In the absence of any other concurrent therapy for her tumor, she remains alive and clinically well with radiographically stable disease after 53 continuous months of disulfiram and Zn^{2+} therapy. Although this represents only a single patient, her survival is unlikely due to chance alone because it greatly exceeds the 7-month median survival seen with ocular melanoma metastatic to liver (40).

One potential mechanism explaining the antiproliferative activity of disulfiram is inhibition of transcription factor DNA binding, which we have shown to be sensitive to disruption by disulfiram in a manner potentiated by heavy metal ions. Melanomas are dependent for growth and metastasis on activation of distinct transcription factors, such as ATF/CREB (36–38) and NF-κB (41). ATF/CREB transcription factors, in particular, play prominent roles in cell proliferation and survival (39, 42), and others have suggested molecular disruption of ATF/CREB-mediated transcription for controlling melanoma growth (37, 38). In addition to having an important cell cycle regulatory function, NF-κB induces expression of several antiapoptotic genes such as *TRAF*, *c-IAP*, *IXAP*, *A1/Bft-1*, and *IEX-1L* (43). Malignancies with constitutive activation of NF-κB such as melanoma (41) or those in which NF-κB is induced by radiation or chemotherapy (43) are resistant to the proapoptotic effects of most current cancer therapies, and strategies that inhibit NF-κB sensitize tumors to chemotherapeutic drugs (43, 44). Similar to our findings that disulfiram increased susceptibility of melanomas to cisplatin or carmustine, others have shown recently that disulfiram increases the susceptibility of human coloretal cancer cell lines to 5-fluorouracil by inhibiting NF-κB (4). In this

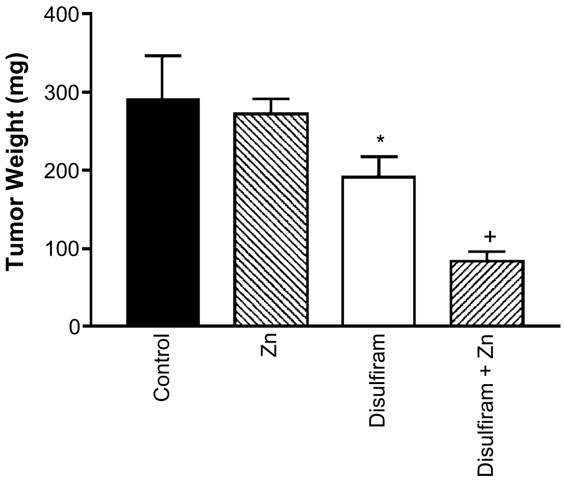

Figure 9. Disulfiram + zinc supplementation decreases malignant melanoma growth in mice. Adult female CB17-SCID mice were injected s.c. in the right groin with 5×10^6 cells from a highly aggressive malignant human melanoma. Mice were fed either a standard diet containing zinc (87 ppm) or a zinc-supplemented diet containing Zn^{2+} (1,000 ppm) as zinc acetate. On the day of tumor injection, all mice began daily oral gavage of olive oil (0.2 mL) as a control or olive oil (0.2 mL) containing the indicated drug. Four groups were studied: tumor control (n = 10; 0.2 mL olive oil daily; standard zinc diet of 87 ppm); zinc-supplemented control (*Zn*; n = 10; 0.2 mL olive oil daily; zinc diet of 1,000 ppm); disulfiram (n = 10; 200 mg/kg/d disulfiram in 0.2 mL olive oil; zinc diet of 87 ppm); and zinc-supplemented diet + disulfiram (n = 10; 200 mg/kg/d disulfiram in 0.2 mL olive oil; zinc diet of 1,000 ppm). When estimated tumor volume in controls approached 500 mm^3, all mice were euthanized and tumors were excised and weighed. Zn^{2+} supplementation alone had no affect on tumor growth, but disulfiram alone and disulfiram + Zn^{2+} supplementation all significantly inhibited tumor growth. *, $P < 0.05$ versus tumors in controls or Zn. +, $P < 0.001$ versus tumors in controls or Zn.

study, Wang et al. (4) found that disulfiram did not prevent degradation of its inhibitor IκB but did inhibit DNA binding of NF-κB *in vitro*. These results agree with our studies showing direct *in vitro* inhibition of ATF/CREB DNA binding in nuclear extracts from melanoma cells in a manner enhanced by cupric ions and glutathione and support our hypothesis that disulfiram disrupts DNA transcription factor binding by forming mixed disulfides that induce *S*-glutathionylation of critical cysteines in the transcription factor DNA binding region. This mechanism has been also suggested for how NO• inhibits the activity of c-Jun, Fos, ATF/CREB, Myb, and Rel/NF-κB family transcription factors (45).

Other important cellular targets for disulfiram also exist, including Cys^{56} of the adenine nucleotide translocator that enforces permeability transition pore complex opening in mitochondria and release of cytochrome *c* and apoptosis inducing factor (14). Mitochondrial permeability transition induced by disulfiram (13) would also be expected to sensitize resistant malignancies to chemotherapeutic treatment (46). However, all of these critical thiols would be expected to behave similarly, with formation of mixed disulfides and perhaps *S*-glutathionylation catalyzed by disulfiram in a fashion enhanced by presence of copper or other similar metal ions. Disulfiram has long been known to form mixed disulfides with cysteine thiols (47). Furthermore, the drug is absorbed as its bis(diethyldithiocarbamato)copper(II) complex (2), further suggesting that a heavy metal-thiolate chelate may be the active drug facilitating mixed disulfide formation. Additional support for a thiolate-metal complex as the proximate effector is provided by results showing that the copper chelator BCPS reduces and medium supplementation with Cu^{2+} or other heavy metal ions facilitates melanoma growth inhibition by disulfiram, that the reducing agent DTT reverses growth inhibition from the thiolate diethyldithiocarbamate, and that X-ray crystallography confirms the structure of dithiocarbamate-metal ion complexes. A growing literature points to cysteine mixed disulfide formation followed by glutathione conjugation as a unique mechanism for regulating activity of cellular proteins (11, 14, 45, 46, 48), including the process by which pyrollidine dithiocarbamate inhibits NF-κB (49). Thus, dithiocarbamate-metal ion complexes might inhibit cellular growth and sensitize tumors to chemotherapy by cysteine modification at multiple sites.

Employed at the currently approved dose of 250 mg/d, disulfiram seems safe and is readily available for application to several novel treatment strategies for malignancies. First, as originally reported by Schreck et al. (50), disulfiram inhibits activation of NF-κB and can could be used to block activation of this transcription factor by conventional cancer chemotherapy, thereby sensitizing tumors to the effects of currently available drugs (4). In this role, disulfiram would be aided by its ability to block the P-glycoprotein extrusion pump used to extrude chemotherapeutic agents from malignant cells (3), another mechanism for tumor drug resistance. However, the systemic levels of disulfiram (10 μmol/L) that might be required to replicate *in vivo* the NF-κB inhibition and chemotherapy sensitization reported by Wang et al. (4) *in vitro* are achievable *in vivo* only with doses of disulfiram (≥500 mg) that produce considerable nausea and other systemic side effects (2). Similar to the experience with our melanoma patient, concurrent supplementation with metal ions might provide NF-κB inhibition using much lower doses of disulfiram. Second, as suggested by Marikovsky et al. (9) and confirmed by Shiah et al. (7), disulfiram might be potentially investigated as an angiogenesis inhibitor. Although its antiangiogenic effect has been postulated recently as complexation by disulfiram of zinc ions from matrix metalloproteinases (7), our results from melanomas transplanted into SCID mice showing fewer vessels in disulfiram + zinc–treated animals (2.0 ± 0.7 vessels per field) compared with those treated with disulfiram alone (2.5 ± 0.7 vessels per field) are not consistent with this mechanism. Furthermore, the disulfiram concentration required to effectively inhibit angiogenesis (5–10 μmol/L) in reported studies (7) can likely be achieved only with relatively high doses in man (2). Concurrent supplementation with metal ions might also lower the effective antiangiogenic dose of disulfiram to one better tolerated. Finally, as shown by our experience with malignant

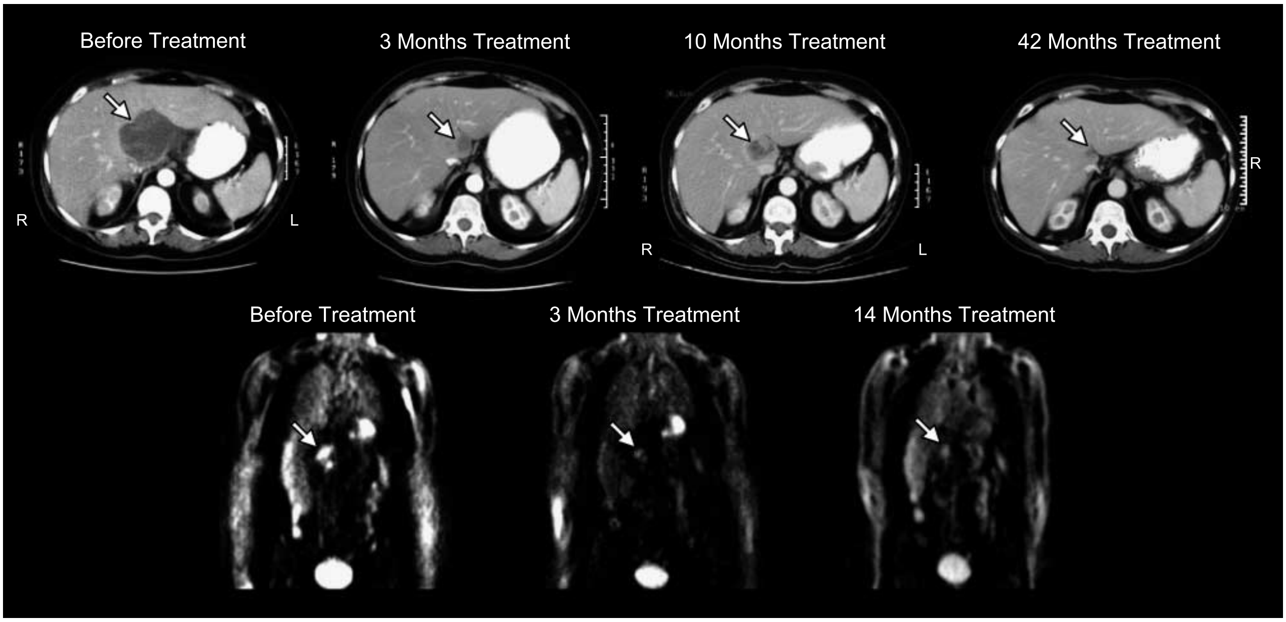

Figure 10. Disulfiram and zinc gluconate reduce hepatic tumor volume in a patient with metastatic ocular melanoma. Computed axial tomograms (*top*) and positron emission spectrographs (*bottom*) of a 64-year-old woman with stage IV ocular melanoma metastatic to the liver. Before treatment, she had a 5.5 cm central liver metastasis (*white arrows*). After 3 months of treatment with disulfiram (500 mg/d) and zinc gluconate (50 mg thrice daily), the hepatic metastasis had decreased in volume by >50% in both scans (*white arrows*). After continuing treatment with disulfiram (250 mg/d) and the same dose of zinc gluconate, the lesion remained stable in size at 10 and 14 months (*white arrows*). She continues to be clinically well and free of drug side effects on disulfiram and zinc gluconate. After 53 continuous months of treatment with this regimen, she has experienced no quantifiable malignant progression. A follow-up abdominal computed tomography scan after 42 months of therapy shows that the hepatic tumor burden has remained small.

melanoma in SCID mice and in the reported patient, disulfiram might offer potential in some tumors as complexing agents for delivery of antiproliferative metal ions to tumor cells. Currently, cisplatin is the only approved metal-based chemotherapy. However, zinc and arsenic are antiproliferative for tumors (16, 51, 52) and zinc and other metals inhibit NF-κB and activator protein-1 (53–56). Therefore, as a potential solution for several current therapeutic difficulties facing clinical oncology, disulfiram may present a novel and readily applicable drug to effect metal-thiolate induced mixed disulfide modification of protein thiols critical for tumor cell drug resistance and growth. In light of our findings and those of others (3–9), we suggest that randomized clinical trials of this strategy are warranted.

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