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 thio carbamate 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 Cu2+ or Zn2+ 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 Cu2+ 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 Zn2+ 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]

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 LD50 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 Zn2+-dependent matrix metalloproteinases (7), or Cu2+ complexation with inactivation of Cu/Zn superoxide dismutase (8, 9) and consequently diminished cellular generation of H2O2 from dismutation of superoxide anion (O2−; refs. 8, 9). Dithiocarbamates
possess a RR’NC(S)SR\textsuperscript{a} functional group, giving them the ability to complex metals (10) and react with sulphydryl groups (10) and glutathione (11). After oxidation to their corresponding disulfides, dithiocarbamates can inhibit critical sulphydryls by forming mixed disulfides with critical cellular thiolis (12), leading to such diverse effects as inhibition of caspases (12) but stimulation of mitochondrial permeability transition (13) and subsequent Bel-independent apoptosis (14). In normal cells, the effects of other dithiocarbamates are potentiated by metals such as Cu\textsuperscript{2+} or Zn\textsuperscript{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\textsubscript{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 thiolis (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(diethylidithiocarbamo)copper(II) complex after passage through the acid environment of the stomach (2). Therefore, Cu\textsuperscript{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\textsubscript{2},N\textsubscript{2}-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\textsuperscript{2+} (provided as CuSO\textsubscript{4}), Zn\textsuperscript{2+} (as ZnCl\textsubscript{2}), Ag\textsuperscript{+} (as silver lactate), or Au\textsuperscript{3+} (as HAuCl\textsubscript{3}/H\textsubscript{2}O) 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\textsubscript{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\textsubscript{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\textsuperscript{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 CuSO4, 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), Cu2+ (0.2–1.6 μmol/L CuSO4), or 1.25 μmol/L disulfiram + various concentrations of Cu2+ 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?) by decomposing 5-nitrosoglutathione (19). NO? could in turn induce mitochondrial permeability transition and apoptosis. To probe whether disulfiram might be inducing growth retardation by altering NO production, proliferation was studied with and without disulfiram in the presence and absence of the NO? synthase inhibitor Nω-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 Cu2+ chelator bathocuproinedisulfonic acid (BCPS, 50 or 100 μmol/L) added to medium to sequester Cu2+ 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’-AGAGATTGGCTGACGTCAGAGAGCTAG-3’ and 3’-TCTCTACGGGTACTTGTTGCTCTCTAGATC-5’ for the CRE and 5’-AGTGGACGAGCTTATCCAGGC-3’ and 3’-CAAATCCCCTGAAAAGGTTCGCG-5’ for NFκB (p50; Promega, Madison, WI). Competition experiments were done with 10× unlabeled wild-type oligonucleotide sequences for CRE or NFκB. 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 succinic 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 mm2 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 Cu2+, 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 medium to sequester Cu2+ in the extracellular compartment. Cells were also treated for 16 hours with disulfiram (100 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).

Measurement of Intracellular Generation of Reactive Oxygen Species

Generation of reactive oxygen species in response to disulfiram with or without CuSO4 was studied using 27-dichlorofluorescin diacetate (Molecular Probes, Eugene,
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Disulfiram (5 μmol/L), with or without 1.6 μmol/L CuSO₄, 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 diethylidithiocarbamate-metal complexes is known in the literature. Typically, aqueous solutions of a metal ion (e.g., CuCl₂) and sodium or ammonium diethylidithiocarbamate are mixed and the desired complex was separated by extraction into an organic phase such as dichloromethane. The stoichiometric ratio between metal ion and diethylidithiocarbamate salt can influence the final stoichiometry of the product. Identical complexes were synthesized starting with disulfiram rather than diethylidithiocarbamate. All diethylidithiocarbamate-metal complexes were characterized by a single crystal X-ray diffraction and structures were reported in the Cambridge Crystallographic Database. The Ag⁺ and Zn²⁺ diethylidithiocarbamate complexes were found to be polymeric. As an example, the results for the Au³⁺ 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 Zn²⁺ as zinc acetate. Mice were injected s.c. in the right groin with 5 × 10⁶ 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); 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 ellipse. When estimated tumor volume approached 500 mm³ 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 (IC₅₀: 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, (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 ellipse. When estimated tumor volume approached 500 mm³ 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.

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 (A₅₄₀). Two-way ANOVA shows P < 0.001 for group and 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.
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% inhibition of growth at 24 hours with 100 ng/mL cisplatin alone versus 17% inhibition with cisplatin and 2.5 μmol/L disulfiram; P < 0.05; 46% stimulation of growth at 24 hours with 10 μmol/L carmustine alone versus 75% 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 for control, 52 for DMSO vehicle, 102 for 1.25 μmol/L disulfiram, 160 for 2.5 μmol/L disulfiram, 195 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 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 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
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Table 1. Effect of complexation or supplementation of cupric ions on antiproliferative activity of disulfiram

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<thead>
<tr>
<th>Treatment</th>
<th>% Growth inhibition</th>
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<tr>
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<td>HTB75 Ovarian Cancer</td>
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<tr>
<td>Disulfiram (0.5 μmol/L)</td>
<td>75 ± 4</td>
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<tr>
<td>Disulfiram (0.5 μmol/L) + BCPS (200 μmol/L)</td>
<td>0 ± 4*</td>
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<tr>
<td>Disulfiram (0.1 μmol/L)</td>
<td>12 ± 4</td>
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<tr>
<td>Disulfiram (0.1 μmol/L) + CuSO4 (0.8 μmol/L)</td>
<td>75 ± 2*</td>
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520 Squamous Lung Cancer 96 Adenosquamous Lung Cancer

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<tr>
<th>Treatment</th>
<th>% Growth inhibition</th>
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<tbody>
<tr>
<td>Disulfiram (0.5 μmol/L)</td>
<td>76 ± 3</td>
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<tr>
<td>Disulfiram (0.5 μmol/L) + BCPS (200 μmol/L)</td>
<td>0 ± 2*</td>
</tr>
<tr>
<td>Disulfiram (0.25 μmol/L)</td>
<td>66 ± 2</td>
</tr>
<tr>
<td>Disulfiram (0.25 μmol/L) + CuSO4 (0.8 μmol/L)</td>
<td>88 ± 2*</td>
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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 Cu2+, the impermeate Cu2+ chelator BCPS was added to medium. To increase the available Cu2+, medium was supplemented with CuSO4. 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.

Figure 5. Disulfiram combined with Cu2+ 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 Cu2+. 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 G0-G1 and G2-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 G2-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 G2-M.
antioxidant probucol did not prevent disulfiram from reducing melanoma proliferation. Augmentation of intracellular copper might also increase levels of NO through Cu$^{2+}$-mediated decomposition of nitrosothiols (19). NO$^*$ might in turn induce mitochondrial permeability transition and apoptosis (26). However, although the NO 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-$\kappa$B 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 $\mu$mol/L disulfiram; 0 ± 0% with 5 $\mu$mol/L silver lactate alone; 71 ± 7% with disulfiram + silver lactate, $P < 0.001$; 0 ± 0% with 5 $\mu$mol/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 diethylthiocarbamate 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 diethylthiocarbamate alone or in the presence of a low concentration of DTT to promote formation of the fully reduced thioacid. Sodium diethylthiocarbamate alone (1 $\mu$mol/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 $\mu$mol/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-$\kappa$B, 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-$\kappa$B (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.
Disulfiram inhibits malignant melanoma

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′-AGAGATTGCAGCTCAGAGACCTG-3′ and 3′-TCTCTAACGGACTGCTCAGTCGCTGATC-5′ for the CRE, and labeled by phosphorylation with [γ-32P]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 Cu2+ 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 CuSO4 (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 + Cu2+ reduced DNA binding by half. *, P < 0.05 compared with other treatments. C, the inhibitory effects of disulfiram or disulfiram + Cu2+ on transcription factor binding are potentiated in the presence of glutathione (GSH). Electrophoretic mobility shift assays were done with addition of disulfiram or disulfiram + CuSO4 (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 Cu2+ 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 Cu2+ 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 + Cu2+). Inhibition of ATF2 containing complex II binding to CRE by disulfiram and Cu2+ in the presence of GSH was reversed by simultaneous addition of the potent uncharged reducing agent DTT (lane 10).

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′-AGAGATTGCAGCTCAGAGACCTG-3′ and 3′-TCTCTAACGGACTGCTCAGTCGCTGATC-5′ for the CRE, and labeled by phosphorylation with [γ-32P]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 Cu2+ 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 CuSO4 (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 + Cu2+ reduced DNA binding by half. *, P < 0.05 compared with other treatments. C, the inhibitory effects of disulfiram or disulfiram + Cu2+ on transcription factor binding are potentiated in the presence of glutathione (GSH). Electrophoretic mobility shift assays were done with addition of disulfiram or disulfiram + CuSO4 (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 Cu2+ 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 Cu2+ 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 + Cu2+). Inhibition of ATF2 containing complex II binding to CRE by disulfiram and Cu2+ in the presence of GSH was reversed by simultaneous addition of the potent uncharged reducing agent DTT (lane 10).

Disulfiram Inhibits Malignant Melanoma

Disulfiram and Zn2+ Inhibit Melanoma Growth and Angiogenesis in Mice

Melanoma cells transplanted into SCID mice grew rapidly as a spherical encapsulated mass. Tumor volume reached ~500 mm3 in controls by 16 days, when animals were sacrificed. Zn2+ alone had no affect on tumor growth (Fig. 9). However, treatment with disulfiram alone or disulfiram + Zn2+ significantly inhibited tumor growth. In mice receiving disulfiram and a Zn2+-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 thio carbamates 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 + Zn2+-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.

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-\(\kappa\)B (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-\(\kappa\)B induces expression of several antiapoptotic genes such as TRAF, c-IAP, IXAP, A1/Bfl-1, and IEX-1L (43). Malignancies with constitutive activation of NF-\(\kappa\)B such as melanoma (41) or those in which NF-\(\kappa\)B is induced by radiation or chemotherapy (43) are resistant to the proapoptotic effects of most current cancer therapies, and strategies that inhibit NF-\(\kappa\)B 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 enhances the susceptibility of human colorectal cancer cell lines to 5-fluorouracil by inhibiting NF-\(\kappa\)B (4). In this

![Figure 8](Image)

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 \(\mu\)mol/L, lanes 5–8), CuSO\(_4\) (Cu, 1.6 \(\mu\)mol/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.
Disulfiram inhibits malignant melanoma

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 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 Shah 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...
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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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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