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Disulfiram Facilitates Intracellular Cu Uptake and Induces Apoptosis in Human Melanoma Cells

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The alcohol-abuse deterrent disulfiram (DSF) is shown to have a highly selective toxicity against melanoma in culture, inducing a largely apoptotic response, with much lower toxicity against several other cell lines. Melanoma cell lines derived from different stages (radial, vertical, and metastatic phase) were all sensitive to DSF treatment in vitro; melanocytes were only slightly affected. A required role of extracellular Cu is demonstrated for DSF toxicity. Low concentrations of DSF alone decreased the number of viable cells, and the addition of $CuCl_2$ significantly enhanced the DSF-induced cell death to less than 10% of control. Significantly, the intracellular Cu concentration of melanoma cells increased rapidly upon DSF treatment. Both the intracellular Cu uptake and the toxicity induced by DSF were blocked by co-incubation with bathocuproine disulfonic acid (BCPD, 100 μM), a non-membrane-permeable Cu chelator. Chemical studies demonstrated a complicated, extracellular redox reaction between Cu(II) and DSF, which forms the complex $Cu(deDTC)_2$ in high yield, accompanied by oxidative decomposition of small amounts of disulfiram. The Cu complex has somewhat higher activity against melanoma and is suggested to be the active agent in DSF-induced toxicity. The redox conversion of DSF was unique to Cu(II) and not engendered by the other common biological metal ions Fe(II or III), Mn(III), and Zn(II). The implications of this work are significant both in the possible treatment of melanoma as well as in limiting the known side-effects of DSF, which we propose may be diminished by cotreatment to decrease adventitious Cu.

Introduction

Dithiocarbamates (DTCs) are sulfur-based chelators that have a broad-spectrum activity against fungal diseases and bacteria and are widely used as pesticides and preservatives.[1] In the last 10 years, a number of groups have demonstrated that various DTCs induce metal uptake and apoptosis in a variety of cells.[2−10] Pyrrolidine dithiocarbamate (pDTC) is the most studied in this regard, as it is commonly used as an antioxidant in cell toxicity studies. Slater and co-workers first demonstrated that pDTC increases oxidative stress in cells due to increased passive uptake of Cu.[2,3] Other reports have confirmed that the cytotoxicity of pDTC is often directly dependent on the availability of metal ions such as Cu and Zn.[4−6] For example, the inhibition of NF-kB by pDTC correlates with increased intracellular Cu levels and can be reversed by high concentrations of non-cell-permeable chelators that compete for the available extracellular Cu.[7] More recently, it has been shown that Cu/pDTC combinations exhibit a specific toxic effect on astrocytes not seen with other divalent transition metals (Mn, Fe, Co, Ni) that can be inhibited by antioxidants or Cu-binding proteins.[8]

Dithiocarbamates and their metabolites are thought to modify intracellular thiols such as cysteine or glutathione and thus inhibit enzymes or disrupt the redox homeostasis in the cells.[11−13] The alcohol-abuse deterrent disulfiram (DSF), a disulfide derivative of *N*,*N*-diethyldithiocarbamate (deDTC), is an inhibitor of aldehyde dehydrogenase, a crucial enzyme in the physiological detoxification of ethanol; the inhibition has been attributed to covalent modification of a cysteine residue in the active site.[14] The toxicity of pDTC has also been proposed to be due to generation of its disulfide form, which can interfere with the glutathione-based redox equilibrium within the cell.[15,16]

There is growing evidence that suggests that the redox regulation within melanoma cells is abnormal.[17] Melanoma cells have a poor ability to mediate oxidative stress compared to normal melanocytes, resulting in an accumulation of reactive oxygen species (ROS) when cells are exposed to glucose oxidase (GOD) generated ROS.[18] The intracellular superoxide level is higher in melanoma cells than in melanocytes.[19] Melanomas also have unusually high uptake of Cu and other metals, even higher than melanocytes, which themselves accumulate metal ions.[20,21]

Several studies have used the abnormal redox-regulation in melanoma cells as a potential target for therapeutic purpose. Melanoma cells are more susceptible to zinc, copper, and cadmium irons at concentrations several orders of magnitudes lower than melanocytes.[21,22] We have previously demonstrated that Cu(II) reacts with melanin to induce ROS formation, and treatment of melanoma cells with either high oxygen (95%) or Cu− or Zn−dithiocarbamate compounds significantly decreases melanoma cell viability, supporting the pro-oxidant effect of these treatments on melanoma cells.[18b] Likewise, DSF induces apoptosis in melanoma

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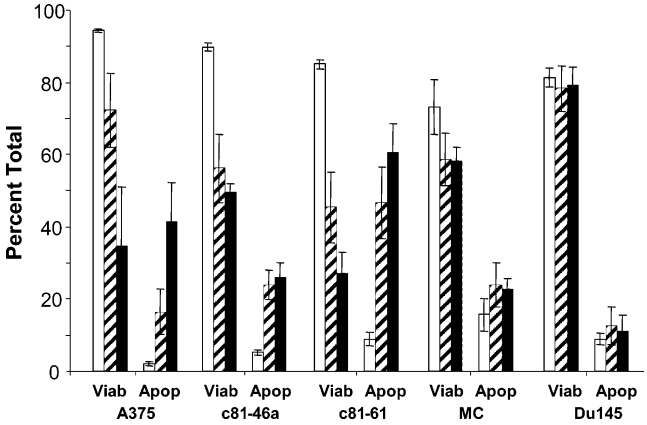
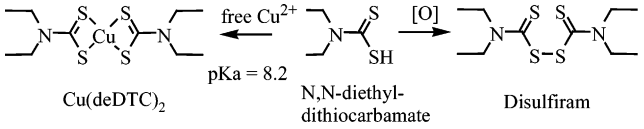


Figure 1. Dosage effects of DSF on melanoma (A375, c81-46a, c81-61), melanocytes (MC), and prostate cancer cell lines (Du145). Bars as percent total cell count: white control, 1 μL DMSO/5 mL media; striped, 25 ng/mL DSF (84 nM); black, 50 ng/mL DSF (168 nM). Cell cultures were dosed over 3 days and stained with Annexin V (AV) and propidium iodide (PI) for analysis by flow-cytometry. Labels: Viab, viable (AV−/PI−); Apop, apoptotic (AV+/PI−). Data adapted from ref 23.

cells and also results in oxidation of cellular glutathione and a disruption of the intracellular redox status.[23] The synergistic effect of Cu^{2+} and disulfiram has also been seen in primary astrocytes cultures, which again is characterized by a dramatic increase in intracellular oxidative stress and apoptosis.[24] Disulfiram is a two-electron oxidized form of diethyldithiocarbamate, a strong Cu chelator, and in this work we give evidence that it is the complex $Cu(deDTC)_2$, shown in Scheme 1,

Scheme 1



which is the causative agent inducing the highly apoptotic response.

Results and Discussion

DSF Toxicity to Melanoma. We have previously shown that the oxidized form of diethyldithiocarbamate, disulfiram or DSF (Scheme 1), induced dramatic increases in apoptosis in several melanoma cell lines.[23] As shown in Figure 1, melanoma cells in culture are much more susceptible than those of normal melanocytes (MC) or prostate cancer (Du145). Especially significant is that a low dose (0.17 μM) caused a steep decrease in melanoma cell viability.

Using the 0.17 μM dose, the toxicities of DSF on melanoma cell lines with different growth patterns, from radial, to vertical, to metastatic stages, were assessed. As shown in Figure 2, the metastatic cell lines A375, c83-2c, c81-46a, and c81-61 were much more sensitive than melanoma cells at the earlier growth stages such as radial M1205 and vertical W3211. At this low dosage, the viability (as determined by AV-/PI- populations) of DSF-treated A375 and c83-2c metastatic melanoma cells was less than one-third of the control, while for the radial stage W3211 cells, the viability was lowered by only 20%. Under these conditions, the viability of normal melanocytes decreased by ca. 15%; note that the overall

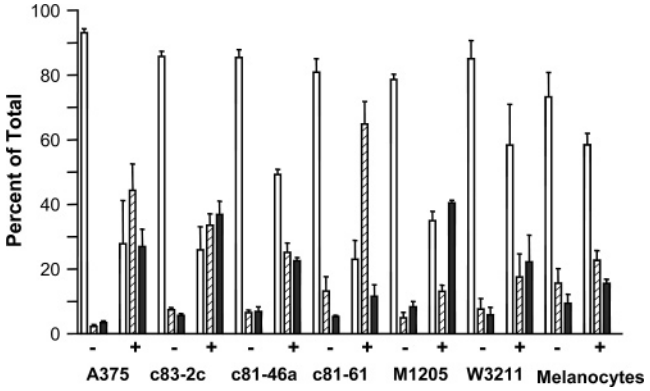


Figure 2. The effect of DSF on melanoma cells. Six melanoma cell lines with varying growth patterns were treated with (+) or without (−) DSF (0.17 μM) for 3 days and stained with Annexin V (AV) and propidium iodide (PI) for analysis by flow cytometry. Bars as the percent total cell count: white bars represent viable (AV−/PI−), striped bars, apoptotic (AV+/PI−); and black bars, necrotic (AV+/PI+). Melanocytes were used as benign controls.

control viability of melanocytes is significantly much lower than that of the cancer cell lines.

Cu Dependence of Toxicity. To study the role of copper in the DSF-induced apoptosis in melanoma cells, melanoma cells (A375 and c81-46a) were exposed to DSF (0.17 μM) and different doses of $CuCl_2$; a non-membrane-permeable copper chelator, bathocuproinedisulfonic acid (BCPD), was used to block the cellular uptake of copper. The toxicity was assayed by both FACs and trypan blue staining; the cytometric assay demonstrated the largely apoptotic response, but gave much lower measures of cell death under equivalent conditions. The FACs analysis assayed a fixed number of cells (10 000) and thus does not judge changes in overall populations or confluency of the cultured cells. Likewise, apoptotic cells that die early in the incubation period may transform into necrotic, as was seen in a study of UV-induced cell death in the human squamous cell carcinoma.[25]

As shown in Figure 3, $CuCl_2$ or BCPD alone did not affect the cell viability. DSF alone reduced the cell viability to 25% of control, and the addition of copper significantly enhanced the effect of DSF. Maximum reduction of cell viability was observed for the addition of 1 μM of $CuCl_2$ (4.2% of control). Co-incubation of BCPD with either DSF or DSF/Cu almost completely blocked their toxicity. The copper-enhancing effect on cell death was also observed in other melanoma cell lines (total viable cell counts were 5% and 5.7% of control for c81-61 and c83-3c, respectively (data not shown).

Using a similar drugging protocol for A375 melanoma cell cultures, the intracellular concentrations of Cu were determined by atomic absorbance measurements of cells lysed after thorough removal of the growth media. The results in Figure 4 are consistent with the hypothesis that the toxicity is Cu dependent. The Cu concentrations are 4-fold higher in the DSF-treated cells and 8-fold higher with addition of $CuCl_2$ to 1 μM. The efficiency of intracellular Cu uptake by DSF is demonstrated by the concentration increases observed: the Cu concentration in the serum-containing culture media is ca. 0.4 μM and increases to 1.4 μM after the addition of $CuCl_2$, roughly proportional to the increased intracellular

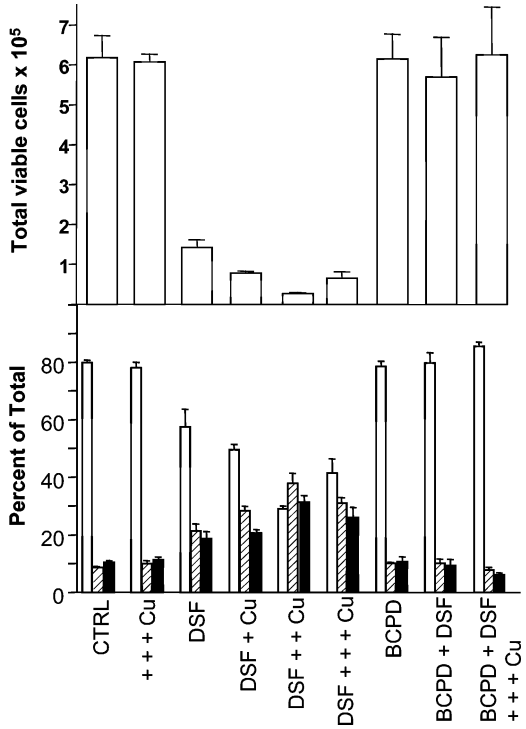


Figure 3. Effect of Cu and a competitive chelator on DSF activity against c81-46a melanoma cells. Top graph, total number of viable cells from Trypan Blue assay. Bottom graph, same conditions as assayed by cell cytometry, with bars as percent total cell count: white bars represent viable (AV−/PI−), striped bars, apoptotic (AV+/PI−); and black bars, necrotic (AV+/PI+). Symbols: CTRL, control, 1 μL DMSO/5 mL media; DSF, disulfiram to 0.17 μM (50 ng/mL); + Cu, 0.1 μM $CuCl_2$; + + Cu, 1.0 μM $CuCl_2$; + + + Cu, 10 μM $CuCl_2$; BCPD, 100 μM bathocuproine disulfonic acid.

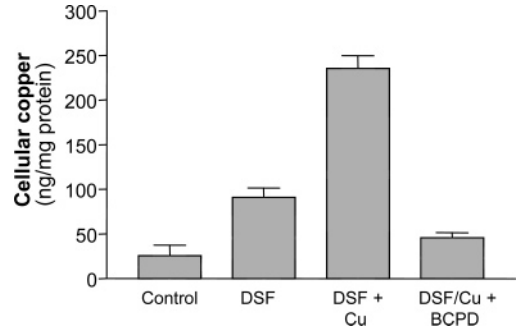


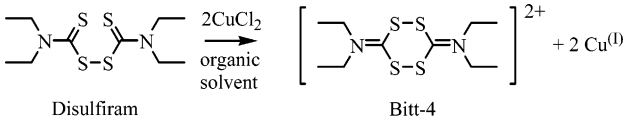
Figure 4. DSF/Cu treatment increased the intracellular copper levels. A375 cells (5×10^7) were treated with DSF (0.17 μM), $CuCl_2$ (1 μM), BCPD (100 μM), or combinations for 4 h, thoroughly washed and lysed, and the lysate assayed by AA for Cu concentration (normalized to protein content). Data (mean ± SEM) are derived from a minimum of three experiments.

concentrations. Significantly, the addition of excess BCPD dramatically lowers the intracellular Cu concentrations to the range of untreated cells, and it likewise decreases the apoptosis response. Thus, the toxicity of DSF to melanoma correlates linearly to the observed increases in intracellular Cu.

Since there was a significant amount of copper (ca. 1 μM) in the serum-containing culture media, we have cultured melanoma cells (A375) in plain DMEM (no copper or zinc). Melanoma cells were incubated with DSF (0.17 μM) with or without copper (1 μM) for 22 h. Only the DSF/Cu combination caused cell death, by both the trypan blue exclusion assay and the apoptosis assay (Supporting Information).

Formation of $Cu(deDTC)_2$ by Reaction of Cu(II) with DSF. Disulfiram is not a traditional chelator like the DTCs, but it and other tetraalkylthiuram disulfides do react with redox active metals such as Cu(II) in organic solvents to give a number of oxidized products.[26,27] As illustrated in Scheme 2, the products are

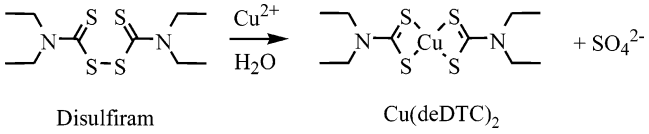
Scheme 2



commonly reduced metal ions and a more highly oxidized form of the DTC, termed a bis(dialkyliminium)-tetrathiolane dication (Bitt-4^{2+}).[28] The reaction of $CuCl_2$ and DSF in anhydrous chloroform is rapid, yielding a red precipitated complex of the stoichiometry [$(CuCl_2)_2$-(Bitt-4)]. In certain solvents, adducts of the reduced DTCs are seen, such as $(deDTC)CuCl_2$.

Surprisingly, the reaction of DSF and $CuCl_2$ in unbuffered aqueous solution directly affords the Cu-$(deDTC)_2$ complex in high yield (Scheme 3). As the

Scheme 3



reaction proceeds in unbuffered solution, the reaction mixture turns a dark brown and the pH drops below 3. The product complex is highly insoluble in water, and following extraction into $CHCl_3$, its formation was readily quantified by a strong absorbance band at 432 nm (Supporting Information). By this method, the yield of $Cu(deDTC)_2$ from a series of stoichiometric reactions of DSF to $CuCl_2$ is ca. 90%. As seen in Table 1, the yield increases only slightly at higher ratios of DSF to Cu-(II), and the excess DSF is readily observable by ESI MS and ^1H NMR. Other identifiable products in the aqueous fraction are sulfate and diethylamine. The pH of the resulting solutions, ca. 2.4, as well as the sulfate produced, ca. 0.05 mmol, vary less than 5% for all reactions tested. Degassing the reaction solution has no effect on the apparent rate of $Cu(deDTC)_2$ formation or on the yield of sulfate byproduct. Significantly, of the bioavailable redox active metal ions (Cu^{2+}, Zn^{2+}, Mn^{2+}, and Fe^{3+}), only Cu yielded a deDTC complex when mixed with DSF in aqueous solution.

DSF is minimally soluble in water and at the concentrations tested the reaction is largely biphasic between soluble Cu(II) and solid DSF. The reaction is strongly inhibited by buffer, such that the yield after 2 weeks in 50 mM iP at pH 7 was only ca. 5% $Cu(deDTC)_2$, seen as black flecks on the glassware and on the insoluble DSF particles. But the addition of a surfactant, dimethyldidodecylammonium bromide (DDAB), at micellar concentration (~10 mM) strongly increased the rate of reaction in pH 7 buffer, giving 45% of the Cu-$(deDTC)_2$ complex after 1 day. The reaction also pro-

Table 1. Analysis of Aqueous Reaction of DSF Plus Metal Salts

reactants	media	conditions	time	% yield[a] complex	% yield[a] SO_4^{2-}	pH
DSF + $CuCl_2$	DI H_2O	aerobic	24 h	89	14	2.4
2× DSF + $CuCl_2$	DI H_2O	aerobic	24 h	70	18	2.4
4× DSF + $CuCl_2$	DI H_2O	aerobic	24 h	93	N/A[d]	N/A
8× DSF + $CuCl_2$	DI H_2O	aerobic	24 h	94	N/A	N/A
DSF + $CuCl_2$	DI H_2O	anaerobic	24 h	91	N/A	2.4
DSF + metal salt[b]	DI H_2O	aerobic	24 h	0	N/A	N/A
DSF	media	aerobic	24 h	25	N/A	N/A
DSF + $CuCl_2$	pH 7.0	aerobic	7 d	4.20	N/A	N/A
DSF + $CuCl_2$	pH 7.0 + DDAB[b]	aerobic	24 h	45	N/A	N/A
[$(CuCl_2)_2$(Bitt-4)]	DI H_2O	aerobic	30 min	29	41	2.8
[$(CuCl_2)_2$(Bitt-4)]	DI H_2O	anaerobic	24 h	33	N/A	2.8

[a] Yields based on molar Cu content. [b] Metal salts tested: $ZnCl_2$, $MnCl_2$, Mn(acetate)$_3$, $FeCl_2$, and $FeCl_3$. [c] DDAB = dimethyldidodecylammonium bromide. [d] N/A = not assayed.

ceeds in biphasic mixture of $CHCl_3$/pH 7 buffer at a slower rate.

To directly test for possible Cu complex formation in the cell viability experiments, 4-fold excess DSF was added to the cell media (ca. 2.6 μM in Cu(II)), and using the same extraction protocol, 82% Cu(deDTC)$_2$ formation is found after 1 day, by EPR analysis (Supporting Information). Thus, the activity of DSF is likely due to its conversion in the cell media to the Cu(deDTC)$_2$ complex.

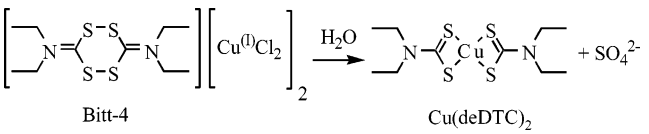
The stoichiometries of these reactions suggest that the decomposition of a small percentage of DSF is sufficient to reduce the remaining DSF to its anionic chelate deDTC form. The balanced equation for a hypothetical decomposition of DSF to diethylamine, sulfate, and carbon dioxide (Scheme 4) demonstrates

Scheme 4

that a high number of reducing equivalents are available from each DSF molecule. The 30 electron equivalents released represent the sum of the change in oxidations states of the four S atoms in DSF (two S^{2-}, two S^{1-}) to that of four sulfates (four S^{6+}). Although the intimate mechanism of such a transformation is not readily apparent, the decomposition a single DSF could, in theory, result in the formation of 15 Cu(deDTC)$_2$ complexes. Under such a scenario, a maximum yield of 93% Cu(deDTC)$_2$ would be obtained in a 1:1 stoichiometric reaction of DSF with $CuCl_2$, with coproduction of about 25% molar yield of sulfate, values not far from the observed yields of 89% and 14%, respectively.

In the aqueous reactions of $CuCl_2$ and DSF, the characteristic red color of the Bitt-4^{2+} species was never observed, but it is possible that these reactions proceed through such an intermediate. The feasibility of Bitt-4^{2+} involvement was demonstrated by hydrolysis of preformed and characterized [$(CuCl_2)_2$(Bitt-4)], which does yielded the Cu(deDTC)$_2$ complex in ca. 30% yield on the basis of Bitt-4^{2+} content (Scheme 5). Again, the

Scheme 5



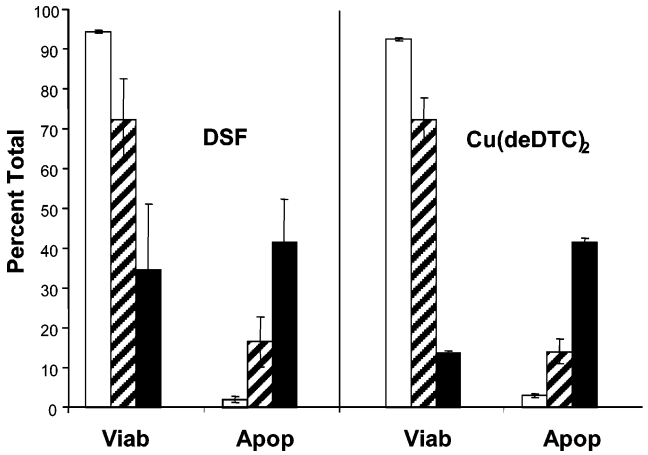


Figure 5. Comparison of the effect of DSF and Cu(deDTC)$_2$ on viability (Viab) and apoptosis (Apop) in melanoma cell line A375. Bars as percent total cell count: white, control, 1 μL DMSO/5 mL media; striped, 25 ng/mL (DSF 84 nM, Cu(deDTC)$_2$ 69 nM); black, 50 ng/mL (DSF 168 nM, Cu(deDTC)$_2$ 138 nM).

aqueous reaction solution is highly acidic, ca. pH 2.6, and the rate and yield of the reaction are not altered by degassing the reaction solutions. Analysis shows that ca. 29% molar yield of sulfate is produced per Bitt-4^{2+}, or ca. 7% of available sulfur. This again suggests an oxidative decomposition, or disproportionation, which generates sufficient reduction equivalents to form the Cu(deDTC)$_2$ complex in fair yield.

Antimelanoma Activity of Cu(DTC)$_2$ Complex. The chemical studies demonstrate that DSF may be converted to Cu(deDTC)$_2$ within cell media prior to uptake by cells in culture and strongly suggest that the antimelanoma active species is in fact attributable to the Cu complex. To test this hypothesis, Cu(deDTC)$_2$ was synthesized independently and its toxicity against melanoma cell line A375 tested (Figure 5). At the lower dosage of 25 ng/μL, the toxicities of DSF and the Cu complex were similar, but note that the actual concentration of the Cu complex, at 68 nM at this dosage, was somewhat lower than that of DSF at 84 nM. At the higher dose of 50 ng/mL Cu(deDTC)$_2$ (138 nM) decreased viability to ca. 15% of control while viability after drugging with DSF at 50 ng/mL (168 nM) is ca. 37%. Thus, the Cu complex has somewhat greater toxicity toward melanoma than DSF, consistent with the substoichiometric conversion of DSF to Cu(deDTC)$_2$.

These results call in to question the varied toxicity of DSF toward other cell lines,[28] which may simply be due

to variations in Cu content between the cell growth media. But DSF has high activity against melanoma cell line A375 and little effect on a prostrate cancer cell line DU145 grown in the same media (Figure 1). Likewise, media-dependent variations should not apply to the toxicity of the Cu complex itself. In this regard, previous studies have shown that both $Cu(pDTC)_2$ and $Zn(pDTC)_2$ complexes demonstrate good antimelanoma activity and that both have increased activity under high oxygen conditions.[18] Most importantly, these complexes have low toxicity toward normal melanocyte cells; at dosages of 100 ng/mL $Cu(pDTC)_2$, melanocyte cultures showed significant enhancements in viability, perhaps due to the antioxidant abilities of the dithiocarbamates. Thus these compounds are selectively toxic to melanomas over normal cells and, in combination with hyperbaric oxygen treatments, may provide a means of selectively targeting metastasized melanoma in vivo.

Conclusions

Disulfiram has been widely and safely used for 50 years, but physiological complications are well-documented for acute DSF intoxication (cerebral seizures and extrapyramidal syndromes), as well as its longterm use (peripheral sensorimotor neuropathy and optic neuritis).[29] It has been shown that neurotoxicity in DSF-treated rats is accompanied by an accumulation of Cu in the hippocampus and cerebellum; this accumulation is suggested as a possible cause of DSF neurotoxicity,[30] and we here have provided a possible chemical mechanism for such Cu accumulation. We would further suggest that DSF-induced neurotoxicity may be limited by a treatment regime that would limit adventitious Cu, as has been used in treatments of Cu-related liver disease.[31]

Previous investigations of the activity of both DSF and other DTC derivatives have focused on biotransformations of the DTC ligand itself, but little consideration was given to what effect metal-uptake might induce.[11,12,15,16] The toxicity of dithiocarbamates such as pDTC have been attributed to a Cu-catalyzed intracellular conversion to a tetraalkylthiuram disulfide (e.g. DSF), which can then disrupt the redox homeostasis within the cell.[15,16] Our results run counter to this scenario in that DSF itself has little effect, but in the presence of Cu(II) it is converted back to the deDTC form. Our evidence points to the $Cu(deDTC)_2$ complex as the active form, but how it then induces the strong apoptotic response remains obscure. In this regard, sulfur oxygenation and extrusion have been proposed as modes of the xenobiotic activation of DSF[11,12] and also as possible sources of its neurotoxicity.[32] We have recently shown that similar sulfur oxygenation and extrusion may occur within a metal−DTC complex.[33] Further studies to identify the chemical mechanism of action and the intracellular targets of $Cu(deDTC)_2$ are under way.

Experimental Section

Abbreviations. DSF, tetraethylthiuram disulfide; deDTC, diethyldithiocarbamate; $Cu(deDTC)_2$, bisdiethyldithiocarbamate−Cu complex; Bitt-4^{2+}, bis(dialkylimonium)tetrathiolane; BCPD, bathocuproine disulfonic acid.

Cell Culture. Metastatic melanoma cells from the A375 cell line were cultured in correspondent DMEM medium (Invitrogen, Grand Island, NY), and c81-46a and c81-61 were cultured in HAM'S F10 (CellGrowth) with 5% fetal calf serum, 5% newborn calf serum, penicillin (100 U/mL, and streptomycin (0.1 mg/mL). Cells were split 1−2 days prior to drug treatment, with a density of $(4-6) \times 10^4$/mL (confluence level of about 75%). Du145 cells were cultured in the same medium as the A375 cells. Melanocytes were processed from pooled neonatal foreskins and cultured in MCDB 153 (Sigma, St Louis, MO) medium containing 2% fetal calf serum, 0.15% bovine pituitary extract (Clonetics, San Diego, CA), 10 ng/mL phorbol myristate-13-acetate, 2 mM calcium chloride, 10 μg/mL insulin, 150 U/mL pencillin, 0.15 mg/mL streptomycin, and 0.1 mM 3-isobutylmethylzanthine (Sigma).

Drug Administration. Drugs were dissolved in the culture medium or in dimethyl sulfoxide and added to the culture directly. Control samples were added with the same amount of medium/or DMSO. The DMSO in the cell media was <0.2%, and previous experiments have shown that DMSO at concentrations below 1% did not affect cell viability.

Apoptosis Assay. Apoptosis assay was performed according to the manufacturer's protocol (Pharmingen). Briefly, cells were trypsinized, washed twice in PBS, and resuspended in binding buffer at a concentration of 1×10^6 cells/mL, of which 100 mL was incubated with 5 mL of Annexin V (AV) conjugated to FITC (Molecular Probes, Eugene, OR) and 75 μM (10 μL) propidium iodide (PI) for 15 min at room temperature. Cells were then analyzed by flow cytometry using a Becton-Dickinson FACScan. The proportion of apoptotic cells was estimated by the percentage of cells that stained positive for AV while remaining impermeable to PI (AV+/PI−), necrosis was defined as a positive stain with both AV and PI (AV+/PI+), and viability was defined as AV−/PI−. Trypan blue stain was also used in parallel, and the percentage of trypan blue positive cells was similar to the sum of the percentages of AV+/PI− and AV+/PI+ cells. This method was based on the previously published literature where, in the early stages of apoptosis, phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane at the cell surface. Annexin V has a high affinity for PS binding on the cell surface. In late stage of cell death, membrane integrity is lost and taken up by propidium iodide.[34]

Measurement of Intracellular Cu. Melanoma cells (A375, $(3-4) \times 10^7$) were treated with DSF (0.17 μM) ± $CuCl_2$ (1 μM), and combinations were treated with BCPD (100 μM) for 4 h at 37 °C. Cell suspension was collected using a rubber policeman. After two washes with PBS, cells were suspended in 0.3 mL of HEPES buffer (15 mM in 0.9% NaCl, pH 7.3). A small aliquot (10 μL) was saved for protein quantification. The rest of the cell suspension was mixed with 0.9 mL of HNO_3 (0.1 N) and incubated at 60 °C for 3−4 h. The resulting cell lysate was measured for Cu content using Spectra AA 250+ machine.

Synthesis of $Cu(S_2CN(C_2H_5)_2)_2$. Bis(diethyldithiocarbamate)Cu(II) was synthesized by a slight modification of literature methods.[35] Sodium diethyldithiocarbamate trihydrate (1.190 g, 5.25 mmol) was added to a stirred light blue solution of $CuCl_2 \cdot 2H_2O$ (0.427 g, 2.56 mmol) in methanol (150 mL) at room temperature. The mixture turned black immediately and was left to stir for 15 min. The resulting precipitant was collected on a glass frit and washed with ice-cold methanol to yielded 0.890 g (96%) of a black solid. Electrospray MS: 359 ($[M]^+$). Anal. Calcd for $H_{20}C_{10}N_2S_4Cu$: C, 33.35; H, 5.59; N, 7.77. Found: C, 33.19; H, 5.56; N, 7.69.

Product Analysis. $Cu(deDTC)_2$ formation was quantified by modification of literature methods, including absorbance and EPR methods (Supporting Information).[36,15] Sulfate was quantified by modification of literature methods (Supporting Information).[36]

Stoichiometric Aqueous Reactions of DSF and $CuCl_2$. Aliquots of 1×, 2×, 4×, and 8× DSF (1 aliquot = 87 mg, 0.2993 mmol) was added to 100 mL of a $CuCl_2$ (50 mg, 0.2993 mmol) solution in deionized water and allowed to stir for 24 h. The

solution is a light blue color upon the initial dissolving of $CuCl_2$ and slowly turns yellow, brown, and then into a colloid black solution as the insoluble black $Cu(deDTC)_2$ is formed. In each preparation, three separate portions of chloroform (100 mLs each) were added to extract all organic soluble products. The absorptivity at 432 nm of each preparation was fit to a calibration curve and gave 89, 70, 93, and 94% yields of $Cu(deDTC)_2$ for the respective stoichiometric reactions. The pH of aqueous fractions from the 1 and 2 equiv reactions of DSF + $CuCl_2$ were both measured to be 2.4, and sulfate assays for these reactions yielded 10.1 and 12.3 mg of $BaSO_4$ (0.04317 and 0.05270 mmol).

Stoichiometric Aqueous Reactions of DSF and Metal Salts (Metal = Zn^{2+}, Mn^{2+}, Mn^{3+} Fe^{2+}, and Fe^{3+}). Each metal salt, $ZnCl_2$, $MnCl_2$, Mn(acetate)$_3$, $FeCl_2$, and $FeCl_3$ (41, 37, 52, 38, and 40 mg, 0.2993 mmol), was stirred in a one-to-one molar ratio with DSF (ca. 87 mg, 0.2993 mmol) in deionized water (100 mL) for 24 h. Each solution had white particles and was colloidal in nature. In each preparation, three separate portions of chloroform (100 mL each) were added to extract all organic soluble products. ^1H and ^{13}C NMR and ESI MS of the chloroform extract showed only DSF (when directly compared to authentic sample of DSF from Aldrich), and no formation of $M(deDTC)_2$ complexes was observed.

Anaerobic Reaction of DSF and $CuCl_2$. The following reaction was prepared in an anaerobic glovebox. To stirring deionized water (~100 mL) was added DSF (87 mg, 0.2993 mmol) along with $CuCl_2$ (50 mg, 0.2993 mmol) and the mixture was allowed to stir for 24 h. The solution is a light blue color upon the intial dissolving of $CuCl_2$ and slowly turns yellow, brown, and then into a colloid black solution as the insoluble black $Cu(deDTC)_2$ is formed. Three separate portions of chloroform (100 mLs each) were added to extract all organic soluble products. The pH of the remaining aqueous fraction was 2.4. The absorptivity assay of the chloroform fraction gave a 91.4% yield of $Cu(deDTC)_2$.

Cell Media Reaction. A solution of DSF (1.2 mg, 0.0404 mmol) was made with 90 mL of Dulbecco's modified eagle medium and 10 mL of fetal bovine serum. The solution was allowed to stir for 1 day. Three separate portions of chloroform (100 mLs each) were added to abstract all organic soluble products. The chloroform was removed in vacuo to yield 264 mg of yellow oil. Other highly colored species in the extract made quantification by the absorbance assay difficult. The EPR assay gave 77 μg or 0.21 μmol of $Cu(deDTC)_2$ or 82% yield based on 2.6 μM Cu^{2+} in media.

Biphasic Reaction. A solution of DSF (0.087 mg, 0.2993 mmol) was prepared in chloroform (~50 mL, 0.2993 mmol) which was slightly yellow in color. Deionized water was slowly added to the reaction, forming two distinct layers. $CuCl_2$ (50 mg) was then added to the water layer, turning it a light blue color. The reactions was allowed to stir for 12 days, in which the chloroform layer slowly turned black upon formation of $Cu(deDTC)_2$. The absorptivity assay gave a 89% yield of $Cu(deDTC)_2$.

Synthesis of [$(CuCl_2)_2$(Bitt-4)]. The complex salt [$(CuCl_2)_2$(Bitt-4)] was made according to literature procedures.[27] A solution of $CuCl_2$ (646 mg, 4.808 mmol) in anhydrous THF (50 mL) was cooled to 0 °C in an ice bath. A solution of DSF (713 mg, 2.404 mmol) in anhydrous CH_2Cl_2 was added dropwise. A brick red solid formed immediately and was filtered, washed with anhydrous pentane (~50 mL), and dried under vacuum to yielded 835 mg (82%) of a red solid. Anal. Calcd for $H_{20}C_{10}N_2S_4Cu_2Cl_4$: C, 21.20; H, 3.63. Found: C, 21.05; H 3.33.

[$(CuCl_2)_2$(Bitt-4)] Hydrolysis. In a 250 mL round-bottom flask [$(CuCl_2)_2$(Bitt-4)] (72 mg, 0.1379 mmol) was added to 150 mL of water in air and stirred vigorously for 30 min. Chloroform was then added to extract all organic soluble products. The absorptivity assay gave a 29% yield of $Cu(deDTC)_2$ based on Cu. The reaction solution was measured to have a pH of 2.8, and sulfate determination gave 13.4 mg or 0.05741 mmol.

Anaerobic [$(CuCl_2)_2$(Bitt-4)] Hydrolysis. The following reaction was prepared in a N_2 glovebox. [$(CuCl_2)_2$(Bitt-4)] (72 mg, 0.1379 mmol) was added to 20 mL of water and the mixture stirred vigorously for 1 day. Chloroform was added to extract all organic soluble products. The pH of the remaining aqueous fraction was 2.8. The absorptivity assay gave a 33% yield of $Cu(deDTC)_2$ based on Cu.

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Supporting Information Available: Data include cell viability assays for melanoma cell line A375 grown in serum-free culture and absorbance and EPR assays for $Cu(deDTC)_2$. This material is available free of charge via the Internet at http://pubs.acs.org.

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