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Nitric Oxide Donors or Nitrite Counteract Copper-[dithiocarbamate]$_2$-Mediated Tumor Cell Death and Inducible Nitric Oxide Synthase Down-Regulation: Possible Role of a Nitrosyl-Copper [Dithiocarbamate]$_2$ Complex

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In contrast to other metal−dithiocarbamate [DEDTC] complexes, the copper−DEDTC complex is highly cytotoxic, inducing oxidative stress, preferentially in tumor cells. Because nitric oxide (NO) forms adducts with Cu[DEDTC]$_2$, we investigated whether NO donors like *S*-nitroso-*N*-acetyl penicillamine (SNAP) or sodium nitroprusside (SNP), and nitrite, a NO decomposition product, modulate Cu[DEDTC]$_2$ cytotoxicity against human tumor cells. We show that apoptosis-associated PARP cleavage and inducible nitric oxide synthase (iNOS) down-regulation induced by nanomolar Cu[DEDTC]$_2$, are counteracted by 50 μM SNAP, SNP, or $CoCl_2$, an inducer of hypoxia and NO signaling. Nitrite was stochiometrically effective in antagonizing Cu[DEDTC]$_2$ cytotoxicity and inducing shifts in the absorption spectrum of the binary complex in the 280 and 450 nm regions. Subtoxic concentrations of Cu[DEDTC]$_2$ became lethal when tumor cells were pretreated with c-PTIO, a membrane-impermeable scavenger for extracellular NO. Our results suggest that: (a) reactive oxygen species induced by Cu[DEDTC]$_2$ are scavenged by nitrite released from NO, (b) the extent of lethality of Cu[DEDTC]$_2$ is dependent on the reciprocal formation of an inactive ternary Cu[DEDTC]$_2$NO copper−nitrosyl complex.

Introduction

Redox regulation is aberrant in tumor cells like melanoma,[1] and redox-modulating agents like synthetic copper(II) complexes can induce cell apoptosis in melanomas[2,3] and breast cancer cells.[4−6] In recent studies, 0.17 μM disulfiram that originates diethyl dithiocarbamate (DEDTC) at the same concentration showed highly selective and significant toxicity against A375 and c81-46a melanoma cells but not normal melanocytes, dependent on addition of 1 μM $CuCl_2$.[2] Moreover, the reaction of disulfiram and $CuCl_2$ in unbuffered aqueous solution directly affords the Cu[DEDTC]$_2$[a] complex in high yield, suggesting that the antimelanoma active species is in fact attributable to the Cu[DEDTC]$_2$ complex.[2,3] In other experiments, MDA-MB-231 cells treated with 20 μmol/L of copper, disulfiram, or both demonstrate apoptosis-associated PARP cleavage and inhibition of proteasome activity when disulfiram and copper were used together.[3] Diethyl dithiocarbamate (DEDTC) also forms complexes with zinc, and 8-hydroxyquinoline (8-OHQ) also complexes with copper.[5] However, in a comparison of the cytotoxic activity of Cu[DEDTC]$_2$, Zn[DEDTC]$_2$, and Cu[8-OHQ]$_2$, we demonstrated that at nanomolar levels, only the copper-[DEDTC]$_2$complex was highly cytotoxic against human wt p53 C8161 melanoma and mutant p53 SKBR3 breast carcinoma without significantly decreasing the viability of normal diploid human WI-38 fibroblasts.[5,6] Lethality induced by nanomolar Cu[DEDTC]$_2$ seems related to oxidative stress because glutathione or catalase counteract such toxicity.[5] Besides the specificity for Cu[DEDTC]2, these results are interesting because tumor cells like SKBR3 frequently show drug resistance due to their mutant p53 status.[5,6] To further learn about the mechanism of Cu[DEDTC]$_2$ toxicity, we investigated its modulation by nitric oxide synthesized by the inducible nitric oxide synthase (iNOS). Constitutive expression of iNOS in many patients' tumor cells, as well as its strong association with poor patient survival, have led to the consideration of iNOS as a molecular marker of poor prognosis as well as a possible target for therapy.[7] The activity of iNOS and the production of nitric oxide end products like nitrite can be inhibited by dithiocarbamates.[8,9] Gaseous nitric oxide also forms stable adducts with copper−dithiocarbamates.[10] Hence, in particular we explored: (a) the cellular effects of Cu[DEDTC]$_2$ on NO production and iNOS expression in human tumor cells, and (b) whether the stochiometric reactivity between nitrite and Cu[DEDTC]$_2$ can modulate the cytotoxic response of the latter. Moreover, because cobalt chloride is important in both hypoxia and iNOS signaling[11] and because there is a link between nitric oxide synthesis and hypoxia-inducible factor-1α,[12,13] we also compared the effects of nitrite and cobalt chloride on the toxicity of Cu[DEDTC]$_2$. Our results showed that iNOS expression and NO are decreased by treatment of melanoma cells with Cu[DEDTC]$_2$ and that these changes

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[a]Abbreviations: Cu[DEDTC]$_2$, copper−dithiocarbamate; NO, nitric oxide; iNOS, inducible nitric oxide synthase; SNAP, *S*-nitroso-*N*-acetyl penicillamine; SNP, sodium nitroprusside; DETA NONOate, diethylenetriamine NONOate.

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were preferentially antagonized by nitric oxide donors or nitrite. Because nitrite diminishes the cytotoxicity of Cu-[DEDTC]$_2$ and also chemically interacts with the binary complex, our data suggests that one mechanism of protection by NO donors may be through nitrite release and formation of a ternary adduct.

Materials and Methods

Preparation and Characterization of Cu[DEDTC]$_2$ and Cu-[DEDTC]$_2$NO Complexes. Sodium dithiocarbamate (DEDTC) was used with copper chloride in a 2:1 [DEDTC−copper] mM ratio, and sodium nitrite was included when the Cu-[DEDTC]$_2$NO was prepared, using the reactants at the 2:1:1 mM ratio of [DEDTC−copper chloride−sodium nitrite]. This copper-dithiocarbamate−NO stoichiometry was compatible with others who previously described formation of a similar complex.[10] The resulting water-insoluble complexes were isolated by filtration, washed, and subsequently redissolved in DMSO. UV−visible spectra of the different compounds were obtained with a UV−vis Milton Roy 2000 spectrophotometer with a diode diffraction array.[5] In some experiments described below, we also compared whether the UV−visible spectrum of Cu[DEDTC]$_2$ was modified by the addition of $CoCl_2$ at the concentrations indicated in the corresponding experiments. Because DEDTC, copper or nitrite at μM levels were soluble in H_2O, we also used mixtures of DEDTC and copper (2:1) or DEDTC, copper and nitrite (2.1:1) in H_2O, whenever indicated. These were compared with the preformed DMSO complexes used at the same micromolar concentration. All reagents used in this study were of the highest purity (≥95%) and were obtained from Sigma Chemical Co., St. Louis, MO) .

Cell Cultures and Treatment. C8161 human melanoma cells were cultured in DME:F12 medium supplemented with 10% fetal bovine serum.[6] Whenever indicated, melanoma cell survival was assessed after exposure to (0.6 μM DEDTC:0.3 μM Cu) unless otherwise specified. Whenever indicated, subconfluent cultures seeded the previous day were treated with nanomolar equivalents of $CuCl_2$ and 2× nanomolar equivalents of diethyl dithiocarbamate [DEDTC]$_2$ to produce Cu[DEDTC]$_2$.

NO Donors. Diethylenetriamine NONOate (DETA NONOate, cat. no. 430-014-M005) was obtained from Alexis Biochemicals, San Diego, CA, *S*-nitroso-*N*-acetyl penicillamine (SNAP, cat. no. N-7892) was obtained from Invitrogen, Eugene, OR. Sodium nitroprusside (SNP, cat. no. 431451), was obtained from Sigma Chemical, St. Louis, MO.

Assesment of Cytotoxicity. Relative cell viability/cytotoxicity was estimated with Alamar Blue (resazurin), which measures intracellular redox activity by quantifying the cell-catalyzed conversion of nonfluorescent resazurin to fluorescent resorufin.[14] When added to a 10% final concentration after the appropriate treatment, this nontoxic dye allows fluorescent quantification and permits reuse for further investigation such as morphological, biochemical, and clonogenic analyses.[14] As such, this assay is valuable as an end point of cytotoxicity rather than as a kinetic measure for monitoring cell growth.[14] For these experiments, cells (10000) were allowed to adhere overnight in 96-well TC microtiter dishes. Following the corresponding treatments, Alamar Blue (BioSource, Camarillo, CA) was added and fluorescence was measured 4 h later in a Fluoroskan Ascent microplate reader with an excitation of 544 nm and an emission of 590 nm.

Colony Formation Assay. Exponentially growing cells were seeded at 5000 cells per well in 96-well plates and were allowed to attach for 18 h. After 48 h of the respective treatments, cells were washed in isotonic phosphate-buffered saline, detached, and transferred to 3.5 cm plates with drug-free complete medium added. Cultures were observed daily for 10−15 days and then were fixed and stained with modified Wright−Giemsa stain (Sigma Chemical, St. Louis, MO). Colonies of multiple cells were scored as survivors.[6]

Immune Blotting and Measurement of Apoptosis. Cell lysates for measurement of cytochrome C release from mitochondria into cytosol were prepared as previously indicated.[6] For all other studies, total cell lysates were used[6] and SDS−PAGE and bidirectional immune blots were done as previously described using 70 μg of proteins per lane,[6] including prestained MW markers (Life Technologies, Gaithersburg, MD) during SDS−PAGE electrophoresis to help identify specific changes in protein expression.After blocking nitrocellulose membranes with 5% skimmed milk in Tris-buffered saline pH 7.5 for 2 h at room temperature, membranes were reacted overnight with specific antibodies in the same blocking solution. Antibodies used for immune blotting included PARP (SC-7150), cytochrome C (SC-13156), iNOS (NOS2) SC-651, all from Santa Cruz Biotechnology (Santa Cruz, CA). Following extensive washing with Tris-buffered saline containing 0.05% Tween 20, membranes were reacted with either anti mouse IgG-peroxidase or protein A-peroxidase, depending on whether primary antibodies were from mouse or rabbit origin. Final detection was achieved by Super Signal-mediated chemiluminescence.[6] For reprobing immune blots, these were incubated in stripping buffer (2%SDS, 62.5 mM Tris−HCl pH 6.8, 0.1 M β-mercaptoethanol) for 30 min at 60 °C, followed by washing the membrane, reblocking, and reacting it with a new set of antibodies. Whenever indicated, Western blots were normalized to total protein loadings, in SDS−PAGE gels stained with Coomassie Blue 0.05% after immune blotting. In other experiments, after stripping of the initial signals as indicated above, reprobing of the immune blots with monoclonal antibody to actin (JLA-20; CPO1) from Oncogene Science (San Diego, CA) was used to normalize protein loadings.

Extracellular and Intracellular NO Determination. Because NO can auto-oxidize in aqueous solution to produce nitrite, extracellular NO was determined fluorometrically upon the reaction of nitrite in cell-free conditioned medium with 2,3-diaminonaphthalene to form the fluorescent product,1-(*H*)-naphthotriazole.[15] For intracellular NO determination, we used the fluorescent dye for NO, 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM/DA) (Invitrogen, Eugene, OR), which emits increased fluorescence after reaction with an active intermediate of NO, formed during the spontaneous oxidation of NO to NO_2^-.[16] Cells were loaded with 5 μmol/L of DAF-FM/DA for 20 min and then rinsed three times. DAF-FM was excited with 490 nm light, and the emitted fluorescence intensities were measured simultaneously at 510 nm. Control experiments were also performed in the presence of the NOS inhibitor, L-NAME (100 μmol/L, Sigma Chemical, St. Louis, MO) to help validate these fluorometric results (not shown).

Statistical Studies. Standard deviations (SD) were computed for all Alamar Blue viability assays, which were carried out in triplicate and repeated four times. In every assay shown, SD results were within ±5% with a 95% statistical significance ($n = 4$, $^{**}p > 0.0001$ by student *t*-test).

Densitometry. Image capture to define differential protein expression was achieved with a Fluor-S Imager (Bio-Rad) followed by quantification of bands with the Gel-Pro software (Media Cybernetics, Silver Spring, MD).

Results

Toxicity Induced by DEDTC-Cu and iNOS Down-Regulation Were Counteracted by Nitric Oxide Donors. Because nitrosative and oxidative stress may counterbalance each other and because Cu[DEDTC]$_2$ can react with nitrite formed by NO* auto-oxidation in aqueous solution,[10] we investigated whether different NO donors were able to counteract the significant cytotoxicty of Cu[DEDTC]$_2$ in human tumor cells.[5,6] Cell viability studies revealed that the cytotoxic effects of aqueous 0.3 μM $CuCl_2$ and 0.6 μM DEDTC in human C8161 melanoma were counteracted by

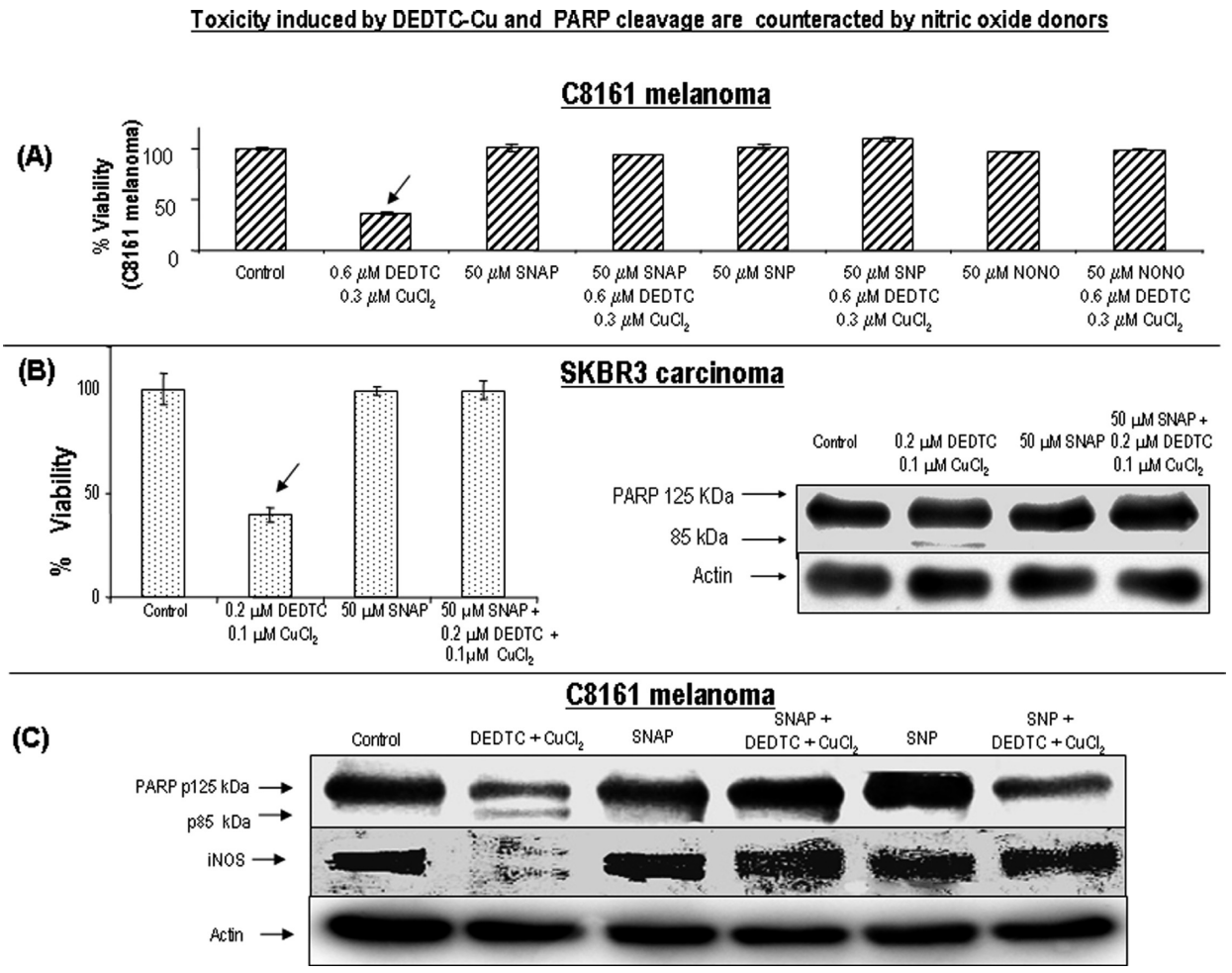


Figure 1. Toxicity induced by $[DEDTC]_2$−Cu and iNOS down-regulation are counteracted by nitric oxide donors. (A) Cell viability assay demonstrates that toxicity induced by 0.6 μM DEDTC and 0.3 μM $CuCl_2$ in C8161 melanoma is antagonized by 50 μM concentrations of NO donors like SNAP, SNP, or DETA NONOate. (B) Cell viability assay demonstrates that toxicity (left) and PARP cleavage induced by 0.2 μM DEDTC and 0.1 μM $CuCl_2$ against SKBR3 carcinoma cells is antagonized by 50 μM SNAP. (C) PARP cleavage and iNOS down-regulation induced by 0.6 μM DEDTC and 0.3 μM $CuCl_2$ are counteracted by NO donors like SNAP or SNP.

6 h pretreatments with 50 μM of either SNAP, SNP, or DETA-NONOate. No difference from control cells was seen with addition of these NO donors at 50 μM in the absence of $CuCl_2$ and DEDTC (Figure 1A). Also, the cytotoxicity of 0.1 μM $CuCl_2$ and 0.2 μM DEDTC against SKBR3 breast carcinoma was counteracted by pretreatment with 50 μM SNAP (Figure 1B, left). These cytotoxicity results were paralleled by the demonstration of apoptosis-associated PARP cleavage[17] in SKBR3 cells treated with $CuCl_2$ and DEDTC and its reversal by 50 μM SNAP (Figure 1B, right). On the basis of the protective effect of NO donors (Figure 1A), the involvement of iNOS in the mechanism for the cytotoxicity of $Cu[DEDTC]_2$ was also investigated.[7−9] Whereas control cultures and those treated with NO donors showed iNOS expression and lack of PARP cleavage, those undergoing PARP fragmentation by the toxic effect of $CuCl_2$ and DEDTC, exhibited loss of iNOS, which was counteracted by cotreatment with SNAP or SNP (Figure 1C).

NO Released from SNP Counteracted PARP Cleavage and Mitochondrial Damage Induced by DEDTC−Copper. SNP is an extremely potent vasodilator used during clinical anesthesia, which behaves as a donor of nitric oxide, cyanide, and iron[18,19] or acts mostly through the release of iron in a Fenton-type reaction after light inactivation at 2 mM levels.[18] We investigated whether 50 μM SNP acted as a NO donor when protecting C8161 melanoma cells from $CuCl_2$ and DEDTC toxicity.[5,6] First, we determined that SNP needed to be present for more than 6 h prior to protect from $CuCl_2$ and DEDTC because adding it for periods of 1 h or less did not prevent its toxicity. We also demonstrated that at 50 μM SNP, the release of iron was not playing a major role in the toxicity of $CuCl_2$ and DEDTC because the iron chelator deferoxamine mesylate (DFO) was not protective (Figure 2A). These results were confirmed with measurements of apoptosis-associated PARP cleavage and the % of cytochrome C released from mitochondria to cytosol,[20] which demonstrated that a brief 30 min pretreatment with SNP did not prevent these changes. However, overnight pretreatment with SNP was quite effective (Figure 2B). The reason for these results were apparent in fluorometric measurements of NO release.[15] These indicated that SNP required at least 6 h to achieve its NO donor potential (Figure 2C) .

Nitric Oxide Scavenger PTIO Potentiated the Effects of Subtoxic DEDTC−Copper. We found that 0.3 μM $CuCl_2$ and 0.6 μM DEDTC were required to induce C8161 melanoma cell death[6] (Figure 1) because use of half these concentrations was ineffective (Figure 3A). However, use of subtoxic concentration [0.15 μM $CuCl_2$ and 0.3 μM DEDTC]

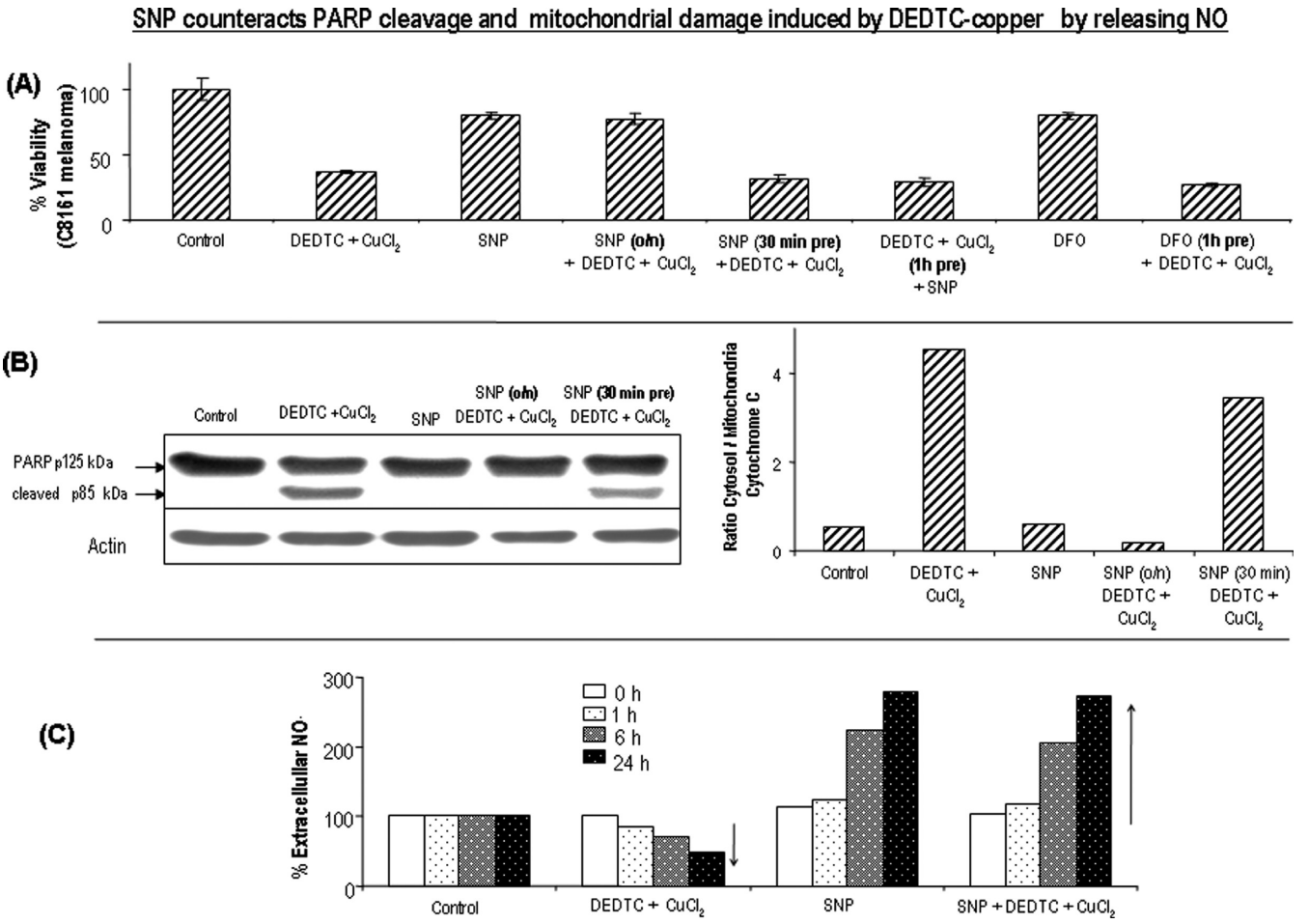


Figure 2. SNP counteracts PARP cleavage and mitochondrial damage induced by DEDTC−copper by releasing NO. (A) Cell viability assay shows that toxicity induced by 0.6 μM DEDTC and 0.3 μM $CuCl_2$ is preferentially antagonized by prolonged SNP overnight (o/n) pretreatment. No comparable protection is afforded by a 30 min pretreatment with SNP, a 1 h treatment with DEDTC + Cu prior to SNP addition, or a 1 h pretreatment with 50 μM deferoxamine (DFO). (B) Overnight (o/n) SNP pretreatment counteracts apoptosis-associated PARP cleavage and mitochondrial damage estimated by cytosolic release of cytochrome C induced by 0.6 μM DEDTC and 0.3 μM $CuCl_2$. (C) Extracellular NO is released from SNP after 6 h of treatment, irrespective of 0.6 μM DEDTC and 0.3 μM $CuCl_2$.

became cytotoxic *only* when cells were pretreated with c-PTIO, a membrane-impermeable scavenger for extracellular NO.[21] Viability assays were compatible with morphological and clonogenic studies (Figure 3A,B) and were confirmed by results showing that 0.15 μM $CuCl_2$ and 0.3 μM DEDTC did not promote apoptosis-associated PARP cleavage[17] unless cells were also treated with c-PTIO, which scavenges extracellular nitric oxide[21] (Figure 3C). We observed a similar potentiation of the effects of 0.15 μM $CuCl_2$ and 0.3 μM DEDTC in subconfluent melanoma cultures when these were added for 16 h after a 2 h pretreatment with 200 μM of the NOS inhibitor, L-NAME (not shown). Taken together, these results suggest an antiapoptotic role for endogenous nitric oxide irrespective of whether it is intracellular or extracellular in human melanoma cells.[8,22]

Modification of the Cu[DEDTC]$_2$ Complex by NO_2 Was Linked to Diminished Cytotoxic Efficacy. Interactions of copper(II) dithiocarbamate with nitric oxide occur in aqueous solution, and decomposition of the latter originates nitrite.[7] Hence, we next investigated the biological and structural consequences of adding sodium nitrite to a solution containing copper(II) and sodium dithiocarbamate, which react to create Cu [DEDTC]$_2$ NO.[10] In cell viability studies, aqueous dithiocarbamate 0.6 μM−copper(II) 0.3 μM (or an equivalent concentration of the preformed Cu[DEDTC]$_2$ complex redissolved in DMSO), had significant cytotoxic effects in C8161 melanoma. However, addition of nitrite 0.3 μM to the aqueous or DMSO-soluble forms of the complex essentially abrogated cytotoxicity, as evident in the binary complex (Figure 4A,B). Nitrite also provided similar protection from cytotoxicity in SKBR3 cells via [dithiocarbamate 0.2 μM−copper(II) 0.1 μM] (not shown). Analysis of the UV−visible spectrum of the ternary Cu-[DEDTC]$_2$NO complex revealed that it differed from the binary Cu[DEDTC]$_2$ spectrum. This confirmed that nitrite originating from nitric oxide decomposition reacted with Cu [DEDTC]$_2$,[7] although no prior study has demonstrated that such interaction alleviates the death-inducing ability of the binary copper complex.

Increases in Nitric Oxide by Cobalt Chloride Were Associated with Protection from DEDTC-Cu Toxicity. Because *S*-nitroso-*N*-acetylpenicillamine (SNAP) stabilizes transactive hypoxia-inducible factor-1α,[12] and this agent also protected from copper(II) dithiocarbamate toxicity (Figure 1), we further investigated whether cobalt chloride, a hypoxia-mimetic agent,[11−13] also protected against Cu[DEDTC]$_2$ toxicity and whether this involved preserving nitrosative status. The cytotoxic effect of Cu [DEDTC]$_2$ was counteracted by

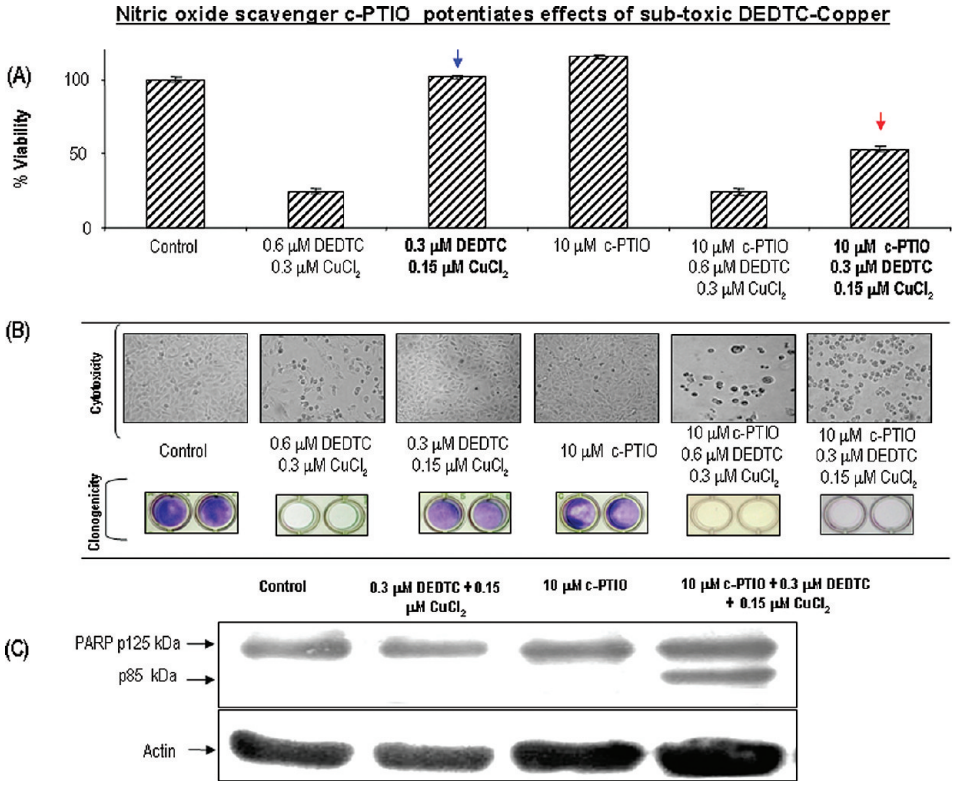
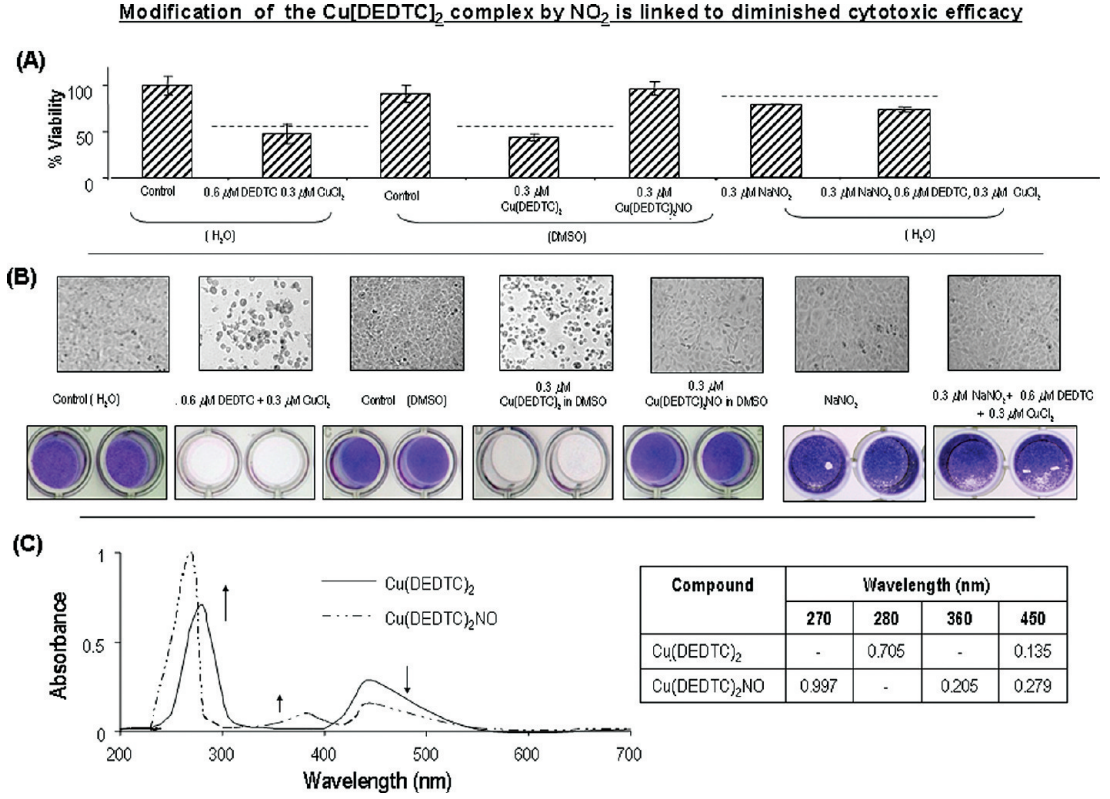


Figure 3. Nitric oxide scavenger PTIO potentiates effects of subtoxic DEDTC−copper. (A) Cell viability assay and (B) clonogenicity assay reveal that the NO scavenger PTIO promotes toxicity of subtoxic 0.3 μM DEDTC and 0.15 μM $CuCl_2$. (C) PTIO enhances apoptosis-associated PARP cleavage induced by subtoxic 0.3 μM DEDTC and 0.15 μM $CuCl_2$.



Compound	Wavelength (nm)			
	270	280	360	450
$Cu(DEDTC)_2$	-	0.705	-	0.135
$Cu(DEDTC)_2NO$	0.997	-	0.205	0.279

Figure 4. Modification of the $Cu[DEDTC]_2$ complex by NO_2 is linked to diminished cytotoxicity. (A) Loss of cell viability induced by aqueous 0.6 μM DEDTC and 0.3 μM $CuCl_2$ or by an equivalent concentration of the preformed $Cu[DEDTC]_2$ complex dissolved in 0.1% DMSO is counteracted by a 90 min pretreatment with 0.3 μM sodium nitrite. (B) Morphological damage and loss of clonogenicity induced by 0.6 μM DEDTC and 0.3 μM $CuCl_2$ or by the preformed $Cu[DEDTC]_2$ complex are counteracted by 0.3 μM sodium nitrite. (C) Addition of nitrite modifies the spectrum of $Cu[DEDTC]_2$.

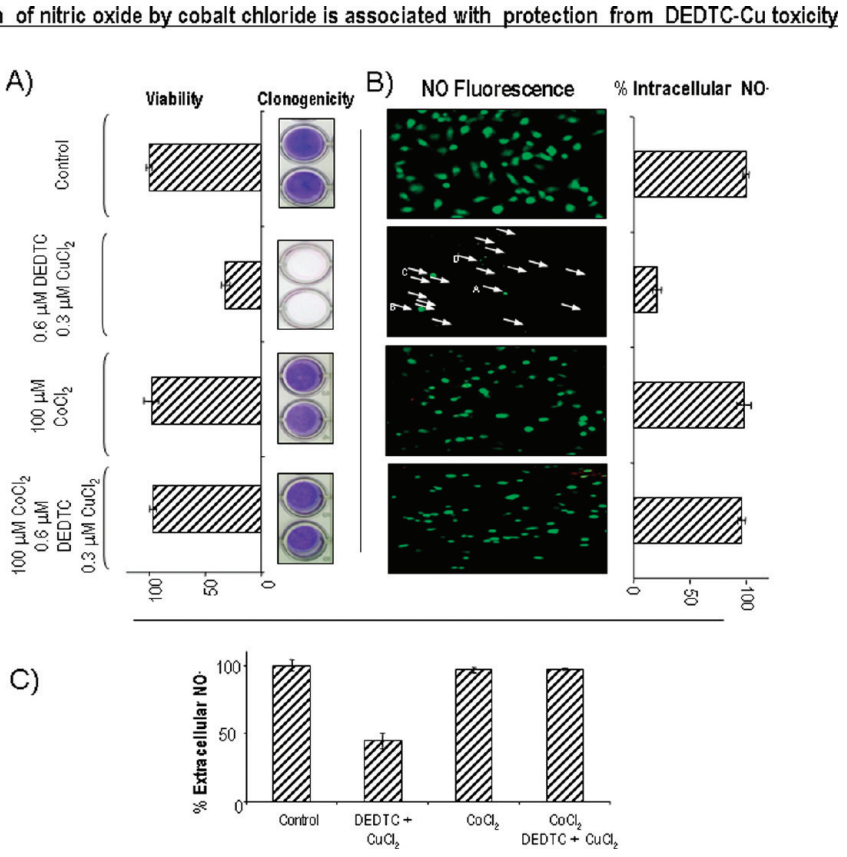


Figure 5. Restoration of nitric oxide by cobalt chloride is associated with protection from DEDTC−Cu toxicity. (A) Aqueous 0.6 μM DEDTC and 0.3 μM $CuCl_2$ induces loss of viability counteracted by overnight pretreatment with 100 μM $CoCl_2$. (B,C) $CoCl_2$ restores loss of intracellular NO and extracellular NO induced by 0.6 μM DEDTC and 0.3 μM $CuCl_2$.

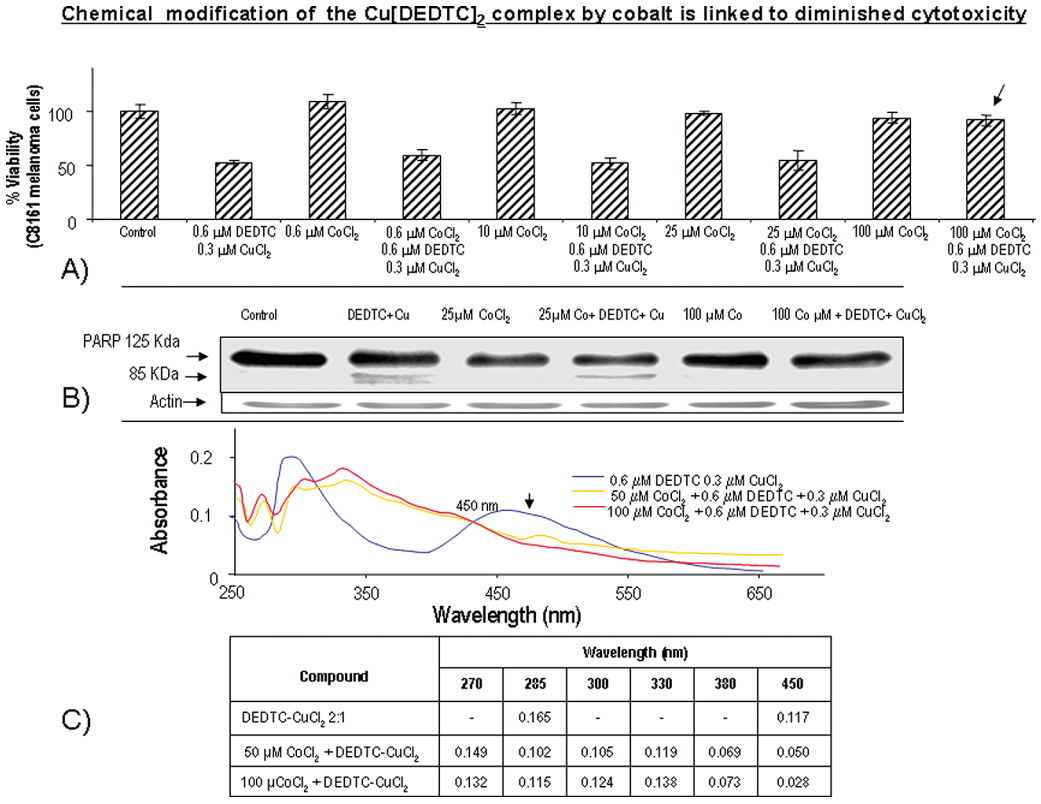
100 μM cobalt chloride (Figure 5A), as revealed by viability and clonogenicity studies. To learn about the involvement of nitric oxide as a mechanism in this protection, we used the NO intracellular indicator DAF-FM/DA.[16] This revealed significant NO reactivity in all cases, except when cells were only treated with Cu $[DEDTC]_2$ (Figure 5B).We confirmed these DAF/FM results showing NO down-regulation by Cu $[DEDTC]_2$ in fluorometric analysis of extracellular NO normalized to cell protein (Figure 5C) by the reaction of nitrite with 2,3-diaminonaphthalene.[15]

Chemical Modification of the Cu$[DEDTC]_2$ Complex by Cobalt Was Linked to Diminished Cytotoxicity. Although cobalt chloride protected from Cu $[DEDTC]_2$ toxicity, it seemed much less effective than nitrite. When we tested different cobalt chloride concentrations, optimal protection was reproducibly achieved between 50 and 100 μM cobalt chloride, as shown by cell viability assays (Figure 6A) and in measurements of apoptosis-associated PARP cleavage (Figure 6B). High cobalt concentrations were required to measurably modify the spectrum of Cu $[DEDTC]_2$ (Figure 6C). These results suggest that these protective effects of cobalt chloride involve preserving nitrosative balance and also cause a structural modification of the copper(II) dithiocarbamate interaction (Figure 6C).

Cobalt Protection from Cu$[DEDTC]_2$ Involved Restoration of iNOS Expression. Finally, we investigated whether the hypoxia-mimetic agent cobalt chloride[11−13] was able to protect other cell types from cytotoxicity induced by the copper(II) dithiocarbamate interaction. Cell viability studies demonstrated that the protection provided by cobalt chloride in C8161 melanoma was also seen in SKBR3 and WM164 cells (Figure 7A). This result was confirmed by clonogenic assays in C8161 melanoma (Figure 7B). Moreover, the iNOS down-regulation accompanying the apoptosis-associated PARP cleavage induced by copper(II) dithiocarbamate was also counteracted by 50 μM cobalt chloride (Figure 7C).

Discussion

In previous work, we demonstrated that cell survival in response to aqueous copper(II) dithiocarbamate or its binary complex involves oxidative stress because *N*-acetylcysteine and hydrogen peroxide-degrading enzymes like exogenous catalase were protective.[5] Our present work demonstrates the constitutive intracellular production of iNOS and NO in viable human C8161 melanoma, as observed in other melanoma cells.[7,22] Although both declined in cells undergoing apoptosis in response to copper(II) dithiocarbamate, these effects were counteracted by nitric oxide donors and by nitrite, the nitric oxide byproduct in aqueous conditions (Figures 1, 2, and 4).These results suggest that dysregulation of the redox balance, caused by a deficiency of antioxidants and/or the overproduction of free radicals, may be the basis for the toxicity induced by Cu$[DEDTC]_2$. However, such oxidative imbalance seems to be counteracted by nitrosative balance, provided by nitric oxide or nitrite.[23] The relevance of nitric oxide metabolism in controlling melanoma survival of copper(II) dithiocarbamate toxicity[7,22] was further highlighted when subtoxic concentrations of these agents became lethal when added to cells pretreated with c-PTIO, a scavenger of extracellular nitric oxide (Figure 3).[21] Our present results also



C)

Compound	Wavelength (nm)					
	270	285	300	330	380	450
DEDTC-$CuCl_2$ 2:1	-	0.165	-	-	-	0.117
50 μM $CoCl_2$ + DEDTC-$CuCl_2$	0.149	0.102	0.105	0.119	0.069	0.050
100 μ$CoCl_2$ + DEDTC-$CuCl_2$	0.132	0.115	0.124	0.138	0.073	0.028

Figure 6. Chemical modification of the $Cu[DEDTC]_2$ complex by cobalt is linked to diminished cytotoxicity. (A) Loss of viability induced by 0.6 μM DEDTC and 0.3 μM $CuCl_2$ is counteracted by pretreatment with 100 μM $CoCl_2$. (B) 100 μM $CoCl_2$ counteracts apoptosis-associated PARP cleavage induced by 0.6 μM DEDTC and 0.3 μM $CuCl_2$, (C) 50 and 100 μM $CoCl_2$ modify the spectrum of $Cu[DEDTC]_2$.

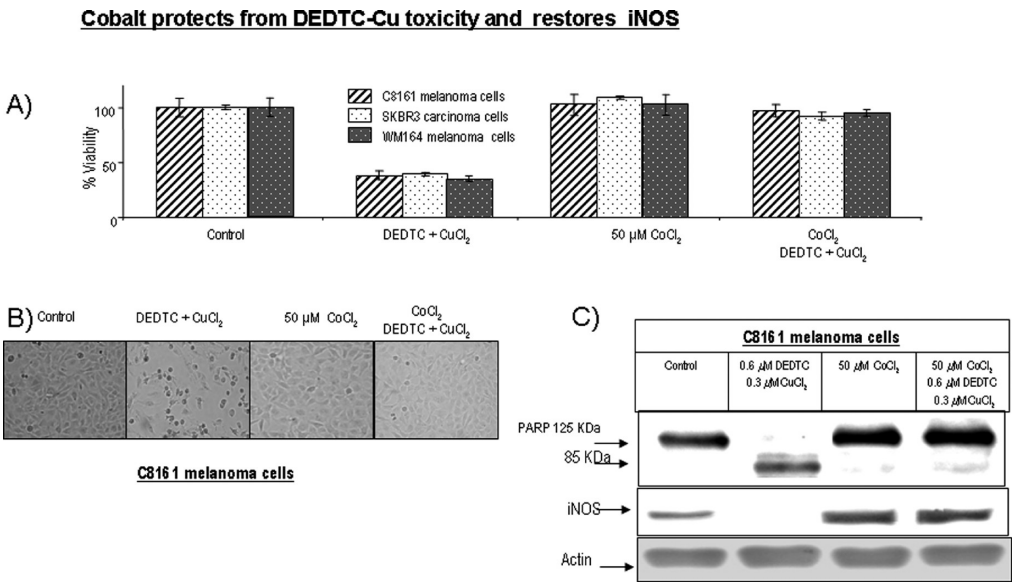


Figure 7. Cobalt protects from DEDTC−Cu toxicity and restores iNOS. (A) Loss of viability induced in C8161 melanoma and WM793 melanoma by 0.6 μM DEDTC and 0.3 μM $CuCl_2$ and in SKBR3 carcinoma by half the concentrations of these agents is counteracted by overnight pretreatment with 50 μM $CoCl_2$. (B) Morphological change induced in C8161 melanoma by 0.6 μM DEDTC and 0.3 μM $CuCl_2$ is counteracted by overnight pretreatment with 50 μM $CoCl_2$. (C) PARP cleavage and loss of iNOS induced by 0.6 μM DEDTC and 0.3 μM $CuCl_2$ in C8161 melanoma are counteracted by overnight pre treatment with 50 μM $CoCl_2$.

suggest a possible link between iNOS and nitric oxide in resistance to apoptosis induced by copper(II) dithiocarbamate. The hypoxia-mimetic agent, cobalt chloride at 50 μM, restored iNOS and nitric oxide from cells treated with copper(II) dithiocarbamate, in agreement with results indicating a link between nitric oxide and hypoxia through HIF-1α.[24] Taken together, increased copper(II) uptake mediated by dithiocarbamate[4−6,25] may cause oxidative stress by forming free radicals (hydroxyl radicals) due to electron transfer when copper shifts from divalent (Cu^{2+}) to monovalent (Cu^{+}).[26] This could lead to greater toxicity when hydrogen peroxide increases intracellularly by induction of endogenous Mn

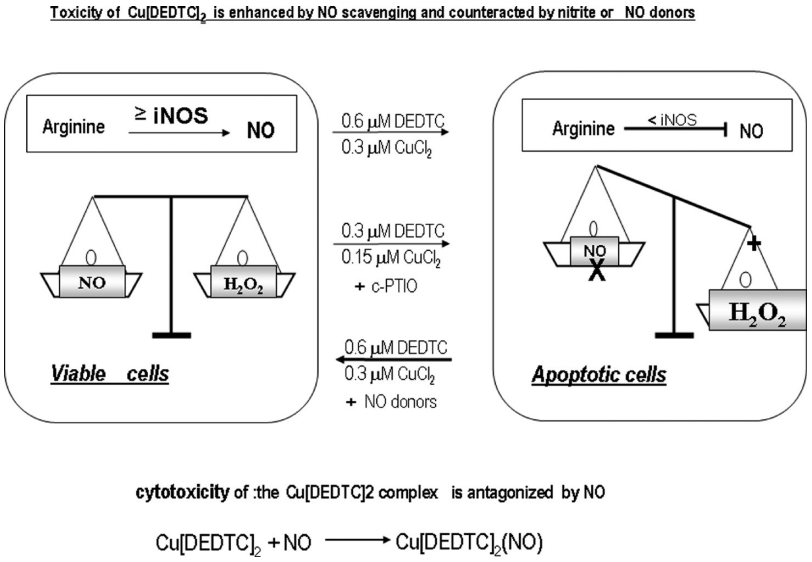


Figure 8. Toxicity of $Cu[DEDTC]_2$ is enhanced by NO scavenging and counteracted by nitrite and NO donors. Toxicity induced by $Cu[DEDTC]_2$ involves iNOS down-regulation is enhanced by c-PTIO scavenging of endogenous NO and is counteracted by NO donors or by nitrite, a product of NO decomposition. Formation of a noncytotoxic $Cu[DEDTC]_2(NO)$ ternary complex is postulated.

superoxide dismutase in response to copper(II) dithiocarbamate.[5,6] In this study, we report for the first time that copper(II) dithiocarbamate treatment also causes nitrosative imbalance,[23] because this lowers production of nitric oxide and down-regulates iNOS, both of which are constitutive in proliferating melanoma[7,22] and other cancer cells.[24] In summary (Figure 8), we propose that melanoma cell survival may require a oxidative−nitrosative equilibrium at basal hydrogen peroxide and nitric oxide levels. Loss of constitutive iNOS and nitric oxide in proliferating melanoma may help to generate toxicity through free radicals.[24−26] Conversely, iNOS and moderate levels of nitric oxide may be important for melanoma survival[8,21] and tumor chemoresistance.[27] Our results with nitric oxide donors, nitrite, and cobalt chloride suggest that copper(II) dithiocarbamate induces apoptosis by decreasing nitric oxide. Similarly, we demonstrate for the first time that ternary complexes of copper(II) dithiocarbamate and nitrite released from nitric oxide restore cell viability, because tumor cells are confronted by less of the biologically active Cu[DEDTC]2 when this becomes inactivated by a chemical modification via coordination of NO to $Cu(DTC)_2$.[10] An interesting question for future studies is whether NO donors, which now show antiapoptotic effects at low concentrations (50−100 μM), may behave as pro-apoptotic agents at higher concentrations[23,27] or in different intracellular milieu.

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References

(1) Meyskens, F. L., Jr.; McNulty, S. E.; Buckmeier, J. A.; Tohidian, N. B.; Spillane, T. J.; Kahlon, R. S.; Gonzalez, R. I. Aberrant redox regulation in human metastatic melanoma cells compared to normal melanocytes. *Free Radical Biol. Med.* **2001**, *31*, 799–808.
(2) Cen, D.; Brayton, D.; Shahandeh, B.; Meyskens, F. L., Jr.; Farmer, P. J. Disulfiram facilitates intracellular Cu uptake and induces apoptosis in human melanoma cells. *J. Med. Chem.* **2004**, *27*, 6914–6920.
(3) Chen, D.; Cui, Q. C.; Yang, H.; Dou, Q. P. Disulfiram, a Clinically Used Anti-Alcoholism Drug and Copper-Binding Agent, Induces Apoptotic Cell Death in Breast Cancer Cultures and Xenografts via Inhibition of the Proteasome Activity. *Cancer Res.* **2006**, *66*, 10425–10433.
(4) Daniel, K. G.; Gupta, P.; Harbach, R. H.; Guida, W. C.; Dou, Q. P. Organic copper complexes as a new class of proteasome inhibitors and apoptosis inducers in human cancer cells. *Biochem. Pharmacol.* **2004**, *67*, 1139–1151.
(5) Viola-Rhenals, M.; Strasberg Rieber, M.; Rieber, M. Suppression of survival in human SKBR3 breast carcinoma in response to metal−chelator complexes is preferential for copper−dithiocarbamate. *Biochem. Pharmacol.* **2006**, *71*, 722–734.
(6) Viola-Rhenals, M.; Strasberg Rieber, M.; Rieber, M. Role of peroxidases, thiols and Bak/Bax in tumor cell susceptibility to $Cu[DEDTC]_2$. *Biochem. Pharmacol.* **2007**, *74*, 841–850.
(7) Grimm, E. A.; Ellerhorst, J.; Tang, C. H.; Ekmekcioglu, S. Constitutive intracellular production of iNOS and NO in human melanoma: possible role in regulation of growth and resistance to apoptosis. *Nitric Oxide* **2008**, *19*, 133–137.
(8) Sherman, M. P.; Aeberhard, E. E.; Wong, V. Z.; Griscavage, J.; Ignarro, L. Pyrrolidine dithiocarbamate inhibits induction of nitric oxide synthase activity in rat alveolar macrophages. *Biochem. Biophys. Res. Commun.* **1993**, *199*, 1301–1308.
(9) Mulsch, A.; Schray-Utz, B.; Mordvintcev, P.; Hauschlidt, S.; Busse, R. Diethyldithiocarbamate inhibits induction of macrophage NO synthase. *FEBS Lett.* **1993**, *321*, 215–218.
(10) Díaz, A.; Ortiz, M.; Sánchez, I.; Cao, R.; Mederos, A.; Sanchiz, J.; Brito, F. Interactions of nitric oxide with copper(II) dithiocarbamates in aqueous solution. *J. Inorg. Biochem.* **2003**, *95*, 283–290.
(11) Xi, L.; Taher, M.; Yin, C.; Salloum, F.; Kukreja, R. C. Cobalt chloride induces delayed cardiac preconditioning in mice through selective activation of HIF-1alpha and AP-1 and iNOS signaling. *Am. J. Physiol. Heart Circ. Physiol.* **2004**, *287*, H2369–H2375.
(12) Park, Y. K.; Ahn, D. R.; Oh, M.; Lee, T.; Yang, E. G.; Son, M.; Park, H. Nitric oxide donor, (±)-*S*-nitroso-*N*-acetylpenicillamine, stabilizes transactive hypoxia-inducible factor-1alpha by inhibiting von Hippel−Lindau recruitment and asparagine hydroxylation. *Mol. Pharmacol.* **2008**, *74*, 236–245.
(13) Jung, F.; Palmer, L. A.; Zhou, N.; Johns, R. A. Hypoxic regulation of inducible nitric oxide synthase via hypoxia inducible factor-1 in cardiac myocytes. *Circ. Res.* **2000**, *86*, 319–325.
(14) O'Brien, J.; Wilson, I.; Orton, T.; Pognan, F. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *Eur. J. Biochem.* **2000**, *267*, 5421–5426.
(15) Cook, J. A.; Kim, S. Y.; Teague, D.; Krishna, M. C.; Pacelli, R.; Mitchell, J. B.; Vodovotz, Y.; Nims, R. W.; Christodoulou, D.; Miles, A. M.; Grisham, M. B.; Wink, D. A. Convenient colorimetric and fluorometric assays for *S*-nitrosothiols. *Anal. Biochem.* **1996**, *238*, 150–158.

(16) Kojima, H.; Nakatsubo, N.; Kikuchi, K.; Kawahara, S.; Kirino, Y.; Nagoshi, H.; Hirata, Y.; Nagano, T. Detection and imaging of nitric oxide with novel fluorescent indicators: diaminofluoresceins. *Anal. Chem.* **1998**, *70*, 2446–2453.
(17) Soldani, C.; Lazzè, M. C.; Bottone, M. G.; Tognon, G.; Biggiogera, M.; Pellicciari, C. E.; Scovassi, A. I. Poly(ADP-ribose) polymerase cleavage during apoptosis: when and where? *Exp. Cell Res.* **2001**, *269*, 193–201.
(18) Cardaci, S.; Filomeni, G.; Rotilio, G.; Ciriolo, M. R. Reactive oxygen species mediate p53 activation and apoptosis induced by sodium nitroprusside in SH-SY5y cells. *Mol. Pharmacol.* **2008**, *74*, 1234–1245.
(19) Gomez-Sarosi, L. A.; Strasberg Rieber, M.; Rieber, M. ERK activation increases nitroprusside induced apoptosis in human melanoma cells irrespective of p53 status: role of superoxide dismutases. *Cancer Biol. Ther.* **2009**, *8*, 1173–1182.
(20) Reddy, P. V.; Rao, K. V.; Norenberg, M. D. The mitochondrial permeability transition, and oxidative and nitrosative stress in the mechanism of copper toxicity in cultured neurons and astrocytes. *Lab. Invest.* **2008**, *88*, 816–830.
(21) Mehta, B.; Begum, G.; Joshi, N. B.; Joshi, P. G. Nitric oxide-mediated modulation of synaptic activity by astrocytic P2Y receptors. *J. Gen. Physiol.* **2008**, *132*, 339–349.
(22) Salvucci, O; Carsana, M; Bersani, I; Tragni, G; Anichini, A. Antiapoptotic role of endogenous nitric oxide in human melanoma cells. *Cancer Res.* **2001**, *61*, 318–326.
(23) Mangialasche, F.; Polidori, M. C.; Monastero, R.; Ercolani, S.; Camarda, C.; Cecchetti, R.; Mecocci, P. Biomarkers of oxidative and nitrosative damage in Alzheimer's disease and mild cognitive impairment. *Ageing Res. Rev.* **2009**, *8*, 285–305.
(24) Quintero, M.; Brennan, P. A.; Thomas, G. J.; Moncada, S. Nitric oxide is a factor in the stabilization of hypoxia-inducible factor-1{alpha} in cancer: Role of free radical formation. *Cancer Res.* **2006**, *66*, 770–774.
(25) Nobel, C. I.; Kimland, M.; Lind, B.; Orrenius, S.; Slater, A. F. Dithiocarbamates induce apoptosis in thymocytes by raising the intracellular level of redox-active copper. *J. Biol. Chem.* **1995**, *270*, 26202–26208.
(26) Gunther, M. R.; Hanna, P. M.; Mason, R. P.; Cohen, M. S. Hydroxyl radical formation from cuprous ion and hydrogen peroxide: a spin-trapping study. *Arch. Biochem. Biophys.* **1995**, *316*, 515–522.
(27) Fetz, V.; Bier, C.; Habtemichael, N.; Schuon, R.; Schweitzer, A.; Kunkel, M.; Engels, K.; Kovács, A. F.; Schneider, S.; Mann, W.; Stauber, R. H.; Knauer, S. K. Inducible NO synthase confers chemoresistance in head and neck cancer by modulating survivin. *Int. J. Cancer* **2009**, *124*, 2033–2041.