Chemical Research in Toxicology

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Ziram and Sodium *N,N*-Dimethyldithiocarbamate Inhibit Ubiquitin Activation through Intracellular Metal Transport and Increased Oxidative Stress in HEK293 Cells

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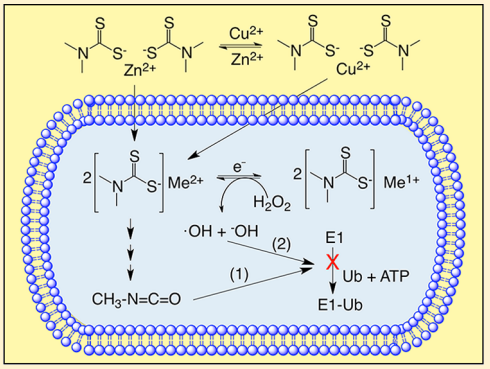
Supporting Information

ABSTRACT: Ubiquitin activating enzyme E1 plays a pivotal role in ubiquitin based protein signaling through regulating the initiating step of the cascade. Previous studies demonstrated that E1 is inhibited by covalent modification of reactive cysteines contained within the ubiquitin-binding groove and by conditions that increase oxidative stress and deplete cellular antioxidants. In this study, we determined the relative contribution of covalent adduction and oxidative stress to E1 inhibition produced by ziram and sodium *N,N*-dimethyldithiocarbamate (DMDC) in HEK293 cells. Although no dithiocarbamate-derived E1 adducts were identified on E1 using shotgun LC/MS/MS for either ziram or DMDC, both dithiocarbamates significantly decreased E1 activity, with ziram demonstrating greater potency. Ziram increased intracellular levels of zinc and copper, DMDC increased intracellular levels of only copper, and both dithiocarbamates enhanced oxidative injury evidenced by elevated levels of protein carbonyls and expression of heme oxygenase-1. To assess the contribution of intracellular copper transport to E1 inhibition, coincubations were performed with the copper chelator triethylenetetramine hydrochloride (TET). TET significantly protected E1 activity for both of the dithiocarbamates and decreased the associated oxidative injury in HEK293 cells as well as prevented dithiocarbamate-mediated lipid peroxidation assayed using an ethyl aracidonate micelle system. Because TET did not completely ameliorate intracellular transport of copper or zinc for ziram, TET apparently maintained E1 activity through its ability to diminish dithiocarbamate-mediated oxidative stress. Experiments to determine the relative contribution of elevated intracellular zinc and copper were performed using a metal free incubation system and showed that increases in either metal were sufficient to inhibit E1. To evaluate the utility of the HEK293 in vitro system for screening environmental agents, a series of additional pesticides and metals was assayed, and eight agents that produced a significant decrease and five that produced a significant increase in activated E1 were identified. These studies suggest that E1 is a sensitive redox sensor that can be modulated by exposure to environmental agents and can regulate downstream cellular processes.



INTRODUCTION

Genetic and genome analyses demonstrate that neurodevelopmental and neurodegenerative disorders are multifactorial in nature and include gene−environment interactions.[1,2] These studies define specific molecular mechanisms and cellular pathways that, when perturbed by multiple gene variants, contribute to neuronal disease or dysfunction. One such essential molecular pathway involves protein ubiquitination, which regulates protein trafficking and activity within the cell and protein degradation through the ubiquitin proteasome system (UPS).[3−5] Protein ubiquitination is particularly important in regulating key activities in the brain, including cellular proliferation, synapse development, neuronal transmission, and synaptic plasticity.[5−7] Genetic mutations and gene variants of proteins in the ubiquitin cascade have been identified as contributing to multifactorial disorders such as Parkinson's disease (PD), suggesting that the nervous system is susceptible to impaired or altered function of protein ubiquitination.[7−10] One singularly important protein is ubiquitin-activating enzyme (E1).[11,12] E1 is the apical enzyme in the enzymatic cascade necessary for protein ubiquitination. E1 catalyzes the initial ATP-dependent thioester formation of ubiquitin to E1 (ub-E1) required for passing ubiquitin on to an E2 conjugase prior to final substrate ubiquitination by either an E2 conjugase or an E3 ligase. Thus, it stands to reason that perturbation of E1 function either through aberrant cellular processes or effects of environmental factors may disrupt a multitude of downstream events dependent upon protein ubiquitination and potentially contribute to the pathogenesis of nervous system disorders.[6,7]

Epidemiological studies support environmental toxin exposure as a risk factor for neurodevelopmental and neurodegenerative diseases.[2,13−15] One recent report suggests that

Received: November 5, 2014
Published: February 25, 2015

DOI: 10.1021/tx500450x
Chem. Res. Toxicol. 2015, 28, 682−690

exposure to certain pesticides, including some dithiocarbamates, increases the risk for PD and that specific genetic variants in genes associated with the UPS can potentiate this risk significantly.[15] Furthermore, animal and cell culture models demonstrate that exposure to environmental toxins can perturb ubiquitin-based protein processing and can be associated with aberrant neurological outcome.[16−18] Importantly, specific deleterious effects of pesticide exposure on activation of E1 in vitro and in vivo have been reported. For example, cultured ventral mesencephalic neurons exposed to ziram, a dithiocarbamate widely used as a fungicide, produced inhibition of E1, loss of tyrosine hydroxylase positive neurons, and elevated α-synuclein levels.[19] Mice administered ziram demonstrated loss of tyrosine hydroxylase positive neurons in the striatum and motor deficits, suggesting that similar effects on E1 may be occurring in vivo. Thus, E1 presents a putative molecular target through which environmental factors can contribute to neurodevelopmental and neurodegenerative diseases.

One potential mechanism of ziram-mediated inhibition of E1 is covalent adduction, a previous study identified E1 as a susceptible target for adduction through both Michael addition and SN_2 mechanisms in HEK293 cell extracts.[20] E1 was specifically adducted on cysteine 234, a cysteine located in the first catalytic cysteine domain (FCCH) of the ubiquitin-binding groove. Subsequent in vivo proteomic studies of rats exposed to *N,N*-diethlyldithiocarbamate (DEDC) identified *S*-ethylaminocarbonyl adducts on brain E1 at Cys234 and Cys179 accompanied by inhibition of E1. Furthermore, rats exposed to DEDC exhibited markers of neurodegenerative change within the nigrostriatal system similar to those present in PD.[21]

While those previous proteomic studies are consistent with inhibition of E1 through electrophilic adduction, other studies report that E1 activity is dependent on the redox status of the cell, suggesting that dithiocarbamates may also inhibit E1 through elevated oxidative stress.[20,21] Dithiocarbamates form metal complexes that vary in their solubility and redox activity depending upon the polarity of the nitrogen substituents and the redox potential of the metal, respectively.[22,23] Dithiocarbamates readily bind copper and dithiocarbamates with nonpolar nitrogen substituents, including DEDC and *N,N*-dimethyldithiocarbamate (DMDC), form redox active copper complexes that cross cell membranes, partition into lipid compartments, and promote oxidative injury.[23−25] This property has been demonstrated in animal models through elevations of copper, lipid peroxidation, protein oxidative injury, and increased apoptosis.[24−26] In the study presented here, we investigated the relative contribution of covalent adduction and oxidative stress in the dithiocarbamate-mediated inhibition of E1 in HEK293 cells.

MATERIALS AND METHODS

Cell Culture and Treatments. HEK293 cells were obtained from American Type Culture Collection (ATCC) bioresource (Manassas, VA) and cultured at 37 °C in 5% CO_2 in Eagle's minimum essential media (EMEM) (ATCC, Manassas, VA) supplemented with 10% fetal calf serum, 100 μg/mL gentamycin, and 2 mM L-glutamine (Gemini Bioproducts, Sacramento, CA) in 75 cm^2 culture flasks until they were 70−80% confluent. Cells were washed once with phosphate buffered saline (PBS), incubated at 37 °C in 1 mL of Accutase (Millipore, Temecula, CA) to detach the cells from the flask surface, and resuspended in 10 mL of media, and the cell concentration was determined with a Scepter 2.0 automated cell counter (Millipore, Temecula, CA). Cells were plated at 1.0×10^6 cells/plate in 100 mm × 20 mm cell culture plates (ThermoScientific, Waltham, MA) and incubated as described above until they were 70−80% confluent. Cultures were supplemented with media as required, and fresh media was added 12 h prior to experimental treatment. Exposure of cells to test compounds was performed as follows: compounds were dissolved in DMSO or distilled water, depending on solubility, and a 1/1000 dilution of each was added to EMEM supplemented as described above. The main compounds utilized in the study were 10 μM ziram (dissolved in DMSO) (Sigma-Aldrich, St. Louis, MO), 20 μM dimethyldithiocarbamate (DMDC) (dissolved in DMSO) (ThermoFisher Scientific, Waltham MA), and either 60 or 120 μM triethylenetetramine hydrochloride (TET) (dissolved in water) (Sigma-Aldrich, St. Louis, MO). All controls contained the equivalent amount of DMSO (0.1%) as experimental cultures, and preliminary experiments were done to confirm that cell viability was unaffected at this concentration. Other compounds utilized are summarized in Table 1, including information on acquisition of the compound. Culture plates were obtained from the incubator, spent media was aspirated, the cells were washed once with 5 mL of PBS, and 8 mL of fresh media containing the designated compound or control was added to the cultures. Plates were returned to the incubator for 6 h and then processed for sample collection.

Table 1. Compounds Tested in HEK293 Cell Culture Assay[a]

category	compound	ub-E1/E1 levels[b]
dimethyldithiocarbamate	10 μM ziram	0.27***
	20 μM DMDC	0.25**
	10 μM thiram	0.67*
thiocarbamate	10 μM molinate	2.5**
dithiocarbamates	20 μM PYDTC	0.57***
	10 μM ZnDEDC	0.32**
	10 μM NaDEDC	0.32**
	10 μM mancozeb	ns
	10 μM maneb	0.22**
	10 μM Me-DETC	ns
	10 μM metam	1.5**
	10 μM Na-DETCSO	ns
	20 μM EPTC	ns
	10 μM disulfiram	ns
	10 μM zineb	ns
	20 μM SADC	ns
	10 μM EITC	ns
benzimadazole	10 μM benomyl	2.3*
triazines	10 μM DACT	3.2***
metal	20 μM $Fe(ClO_4)_2$	1.8*
	20 μM $MnCl_2$	0.36**
E1 inhibiter	20 μM Pyr-41	0.40**
other	4-hydroxynonenal	3.2*

[a]HEK293 cells were incubated in the presence of the above compounds for 6 h as described in the Materials and Methods. The mean ratio of ub-E1/E1 values (+SE) for each treatment group was calculated and compared to control, using one-way ANOVA and Tukey's multiple comparison test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.0001$; $n = 5$ for all groups). [b]The level of ub-E1 inhibition is calculated as follows: (OD ub-E1/$E1_{compound}$)/(OD ubE1/$E1_{control}$). PYDTC, pyrrolidine dithiocarbamate; ZnDEDC, zinc diethlydithiocarbamate; NaDEDC, sodium diethyldithiocarbamate; EPTC, *s*-ethyl dipropylthiocarbamate; SADC, sarcosine dithiocarbamate; DACT, diaminochlorotriazine; ns, not significant

Preparation of Protein Extracts and Determination of Protein Concentrations. Individual plates were washed once with PBS, and 0.5 mL of Accutase was applied to detach cells from the plate's surface. Following a short incubation at 37 °C in 5% CO_2, the plates were retrieved, and the cells were resuspended in 10 mL of PBS, triturated to a single cell suspension, and transferred to a 15 mL conical tube. An aliquot of the cell suspension was removed to

DOI: 10.1021/tx500450x
Chem. Res. Toxicol. 2015, 28, 682−690

determine cell viability using the Sceptor 2.0. Cells were exposed to compounds for 6 h, and cultures were used only when no significant differences in viability existed between controls and treated samples and only when their viability was 85% or better. The cell suspension was subjected to centrifugation at 400*g* for 5 min. The supernatant was aspirated from the tube, and the cell pellet was lysed with the addition of a protein extraction buffer containing 50 mM citrate, 150 mM NaCl, 0.2% NP-40, 3 mM EDTA, pH 4.6, and protease and phosphatase inhibitor cocktail tablets (Roche Diagnostics, Manneheim, Germany) at recommended concentrations. Extracts were subjected to centrifugation at 21 000*g* for 30 min to pellet cell membrane debris, and the supernatant containing cytosolic and nuclear proteins was retrieved and transferred to a clean tube. Extracts were snap-frozen on dry ice and placed at −80 °C for storage. Protein concentrations were determined using the modified Lowry protein or BSA assay kits (Thermo Scientific, Waltham, MA).

Analysis of Activated E1 Protein. The levels of ub-E1 were detected using an established method.[21] Five micrograms of protein extracts from treated and untreated cell lysates was subjected to SDS-PAGE gel electrophoresis under nondenaturing conditions, and the separated proteins were transferred to PVDF membranes. Membranes were blocked in phosphate buffered saline containing 0.05% Tween-20 (PBST) and 5% bovine serum albumin (BSA) and subsequently incubated with a primary rabbit polyclonal antibody against E1 (PW8385, Enzo Life Sciences, Plymouth Meeting, PA). Membranes were washed three times with PBST and incubated with an anti-rabbit polyclonal secondary antibody conjugated to horseradish peroxidase (HRP) diluted in PBST with 1% BSA for 1 to 2 h (Sigma-Aldrich, St. Louis, MO). Final washes were performed, and ubE1/E1 was visualized as two bands of approximate molecular weights of 110 and 117 kDa using Pierce enhanced chemiluminescence (ECL) western blotting substrate for chemiluminescence detection (Thermo-Scientific, Waltham, MA) as directed. HyBlot CL autoradiography films (Danville Scientific, Matuchen, NJ) were exposed to the treated blots, and developed films were scanned with a GS-700 densitometer. The data was analyzed using the Quantity One 1D analysis program, version 4.1 (Bio-Rad, Hercules, CA). The ratio of the activated higher molecular weight ub-E1 species to the nonactivated lower molecular weight species was calculated using densitometry. Comparisons of the mean ratios for the two groups were performed using the unpaired *t*-test.

Immunoblot Procedures for Detection of Heme-oxygenase 1 (HO-1) and Total E1. The appropriate amount of protein extract necessary for detection of each protein was determined empirically using western blot analysis. Briefly, protein extract (5−30 μg) was diluted in LDS sample buffer (Life Technologies, Carlsbad, CA) containing 50 mM dithiothreitol (DTT), heated at 70 °C for 10 min, and subjected to SDS-PAGE gel electrophoresis. The separated proteins were transferred to PVDF membranes (Millipore, Temecula, CA) using the semidry blotting method (Invitrogen, Carlsbad, CA), and the membranes were blocked in PBS containing 0.05% Tween-20 (PBST) and 5% BSA. The blots were subsequently incubated with primary antibodies for the protein of interest, including rabbit anti-E1 and mouse anti-HO-1 (Enzo Life Sciences, Plymouth Meeting, PA). Membranes were washed three times with PBST and incubated with the appropriate secondary antibodies (goat anti-rabbit-HRP or goat anti-mouse-HRP, 1/1000 dilution, Sigma-Aldrich, St. Louis, MO) diluted in PBST with 1% BSA for 1 to 2 h. Final washes were performed, and proteins were visualized and analyzed as described above.

Isolation of E1 and Characterization of Covalent Modifications by LC/MS/MS. Affinity binding and cross-linking of E1 protein to Dynabeads with protein G (Dynabeads) (Life Technologies, Carlsbad, CA) was accomplished as follows: Dynabeads were resuspended, and 7.5 mg of the bead slurry was transferred to a microfuge tube. The beads were washed three times with 500 μL of 0.1 M citrate/phosphate buffer, and a solution of 50 μg of a rabbit polyclonal antibody (Bethyl Laboratories, Montgomery, TX) in 200 μL of citrate/phosphate buffer was prepared and added to the beads following the washes. The mixture was incubated for 3 h at 4 °C to allow for affinity binding of the antibody to the beads. Following the incubation, the bead/antibody mixture was washed three times with citrate/phosphate buffer and with two subsequent washes with 20 mM triethanolamine, pH 8.2 (TEA). Subsequently, 250 μL of a 20 mM solution of the imidoester cross-linker dimethyl pimelimidate·2HCl (DMP) (ThermoScientific) in 20 mM TEA was added, and the mixture was incubated for 30 min at room temperature with rocking. The DMP solution was removed, and the cross-linked bead/antibody mixture was washed for 15 min in 50 mM Tris, pH 7.5, at room temperature followed by three washes with PBST and then incubation in 250 μL of 0.1 M glycine for 5 min at room temperature. Three more washes with PBST were applied, and the bead/antibody mixture was collected, resuspended in 250 μL of PBST, and stored until use at 4 °C. For the immunoprecipitation procedure, the prepared antibody/bead mixture was resuspended, and 25 μL of the slurry was transferred into microfuge tubes for each sample and washed three times with 500 μL of PBST. The last wash was removed from the beads, and 100 μg of cell protein extract in 250 μL of PBST was added to the beads. The mixture was incubated at 4 °C for 3 h with rotation. The beads were washed three times with PBST and resuspended in 20 μL of 1% formic acid, the solution was evaporated using the SpeedVac (GMI, Ramsey, MN), and the dried proteins were stored at 4 °C until processed. To determine covalent protein modifications, the dried proteins were first resuspended in a solution of 8 M urea and 100 mM Tris, pH 8.5, and reduced with TCEP (10 mM). Sample was diluted back to 2 M urea, trypsin was added, and digestion was allowed to proceed overnight at 37 °C. Data on resulting peptides were acquired by first separating them on a 20 cm × 0.1 mm RP analytical column packed into a nanospray emitter tip directly coupled to an Q-Exactive mass spectrometry (ThermoFisher) using an aqueous (A: 0.1% formic acid) to organic (B: acetonitrile, 0.1% formic acid) gradient delivered by an Eksigent HPLC pump at 400 nL/min. Starting gradient conditions were 98% A and 2% B, and after a 10 min load phase, B was ramped to 40% over 70 min and was then further ramped to 95% over 5 min, held for 1 min, and then returned to 98% A for the remainder of the 90 min cycle. Peptides were ionized directly into the mass spectrometry, where both intact masses (MS) and fragmentation patterns (MS/MS) of the peptides were collected. Peptide MS/MS spectral data were searched against the protein database using Sequest,[27] and identifications were collated and filtered using Scaffold (Proteosome Software Inc., Portland, OR).

Determination of Protein Carbonyl Content. Carbonylated proteins were quantified using a published method.[28] Protein (2.5−5.0 μg) in 5 μL of lysis buffer was added to 5 μL of 12% sodium dodecyl sulfate and 10 μL of 20 mM 2,4-dinitrophenylhydrazine (DNPH) in 1,1,1-trichloroacetic acid. The solution was vortexed and incubated at 25 °C for 20 min, and then 7.5 μL of neutralizing solution was added (2 M Tris, 30% glycerol, 19% 2-mercaptoethanol). Negative protein controls were prepared similarly except that 1,1,1-trichloroacetic acid without DNPH was used. Equal amounts of DNPH derivatized and negative control protein samples were loaded, separated by SDS-PAGE, and transferred to Immobilon-P membrane using an XCell II Blot Module (Invitrogen, Carlsbad, CA). After blocking nonspecific binding sites, the membranes were probed with a 1:10000 dilution of a rabbit monoclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (2118S, Cell Signaling Technology Inc., Danvers, MA), and GAPDH was measured by densitometry after incubation using horseradish peroxidase conjugated secondary antibody (A-8275, anti-rabbit-HRP, Sigma, St. Louis, MO, dilution 1:20000) and chemiluminescence. The membranes were then probed using polyclonal rabbit anti-DNPH antibody (A-6430: Molecular Probes, Eugene, OR, dilution 1:10000), and the carbonylated proteins were measured using densitometry after incubation with HRP-conjugated anti-rabbit secondary antibody and chemiluminescence. The optical density of carbonylated proteins for each sample was normalized to the optical density of GAPDH within the same sample.

In Vitro Dithiocarbamate Incubations and Malondialdehyde (MDA) Analysis. An emulsion of ethyl arachidonate in 0.1 M phosphate buffer containing 0.2% SDS (pH 7.4) was prepared by removing the solvent from a 100 mM solution in chloroform (80 μL)

DOI: 10.1021/tx500450x
Chem. Res. Toxicol. 2015, 28, 682−690

and sonicating the residue in the same buffer (1 mL) for 5 min. An aliquot (150 μL) of the suspension was mixed with 1 mM $CuCl_2$ 20 mM ascorbic acid and phosphate buffer (pH 7.4) with ziram or DMDC alone or combined with triethylenetetramine hydrochloride (TET) in the same buffer. The final concentrations were 4 mM ethyl arachidonate, 4 mM ascorbic acid, 100 μM Cu^{2+}, 50 μM ziram, 100 μM DMDC, and 600 μM TET. Controls were incubated without dithiocarbamate or TET. The samples were incubated at 37 °C for 2 h and centrifuged at 10 000*g* for 10 min. Next, 100 μL aliquots of supernatant were mixed with 100 μL of 2,6-di-*tert*-butyl-4-methyl-phenol (BHT) in ethanol (20 mM) and 800 μL of diethyl thiobarbiturate acid (DETBA) reagent (100 mg of DETBA was dissolved in 10 mL of warm ethanol and mixed with 40 mL of 0.125 M phosphate buffer (pH 3.0)) and incubated at 95 °C for 1 h. The solutions were cooled to room temperature, then purified by passing through 1 mL Supelclean LC-18 SPE cartridges (Supelco, Inc., Bellefonte, PA), eluted with methanol, and dried. The residue was reconstituted with 100 μL of a 1:1 mix of eluent A and B. Eluent A was 10% acetonitrile with 0.1% triethanolamine and eluent B was 100% acetonitrile. After filtration through 0.22 μm SPIN-X filters (Corning Inc., Corning, NY), the samples were analyzed using a LichroCART 125 × 4 mm Lichrosphere 100 RP-18 (5 μm) column (EM Science, Gibbstown, NJ) running at 1 mL/min. The elution profile was 100% A going to 90% B in 7 min, holding for 5 min, and returning to A in 1 min. A Shimadzu LC-10AD pump connected to a SIL-10AD autosampler and SPD-10A UV/vis detector controlled by a SLC-10A controller and EZStart 7.4 software were used for the analysis. Under these conditions, the MDA adduct was detected at 530 nm and eluted at 8.5 min. Standard solutions of 1,1,3,3-tetramethyoxypropane (TMP), precursor to MDA, in a 1:1 mixture of ethanol and water were made and reacted with DETBA reagent to generate a MDA standard curve.

ICPMS Measurement of Intracellular Metals. One hundred and twenty micrograms of protein extract was digested overnight in 1 mL of 50% Omni*Trace* Ultra nitric acid in metal-free conical tubes (VWR, Radnor, PA) and then diluted with 9 mL of Millipore high-resistance purified water. Element quantification analysis was performed with Thermo Element 2 HR-ICPMS (Thermo Fisher Scientific, Bremen, Germany) equipped with the ESI autosampler (Elemental Scientific, Omaha, NE). The diluted acid digested samples were taken up through automated aspiration via a 0.50 mm i.d. sample probe and capillary. Elements of interest were measured on isotopes ^{55}Mn, ^{56}Fe, ^{63}Cu, and ^{66}Zn at medium resolution (R = 4300) to separate any potential molecular interference.

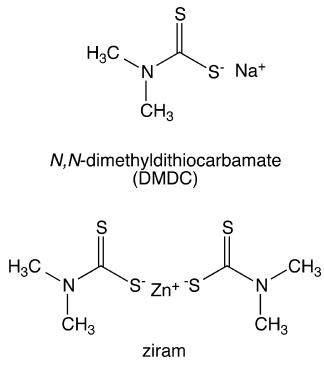
Statistical Analyses. One-tailed and two-tailed Student's t tests, one-way ANOVA, Tukey's multiple comparison test, and Dunnett's test were performed using Prism 4 (GraphPad Software, Inc.). For proteomics expression comparisons, Decyder software, version 6.5 (GE Healthcare Bio-Sciences) was used. Statistical significance was taken to be $p < 0.05$ unless otherwise noted. Treatment groups consisted of $n = 5$ unless otherwise noted.

RESULTS AND DISCUSSION

Previous studies demonstrated that E1 was covalently modified at reactive cysteines within the ubiquitin-binding groove in brain tissues of rats treated with DEDC and in HEK293 cell extracts exposed to thiol-reactive electrophiles.[20,21] To determine if E1 is a target for electrophilic adduction by other dithiocarbamates, we used an in vitro cell culture exposure assay followed by western blot analysis to quantify ub-E1. We used HEK293 cells because they offer a robust human cell line amenable to studying the multiple incubation conditions employed in this study and because they are a previously established model for evaluating covalent adduction of E1 by thiol-reactive electrophiles and ziram-mediated inhibition of E1.[19,20] Subsequent to establishing a decrease in ub-E1 in our samples, E1−enriched cell extracts were subjected to LC/MS/MS for detection of dithiocarbamate-derived adducts.

We utilized two dithiocarbamates, ziram and DMDC (Scheme 1), in these studies, as the structure and potential

Scheme 1



adducts formed by these compounds would be comparable to those for DEDC.[21] Ziram at 10 μM produced a 75% reduction in ub-E1, and 20 μM DMDC significantly decreased ub-E1 to almost half that of the controls (Figure 1); ziram also significantly inhibited E1 at 1 μM (Supporting Information). To assess whether altered expression of E1 contributed to the changes in the ratio of ub-E1 to E1, we determined total E1 levels in our treated cells and found that they remained unchanged (data not shown). To determine if covalent

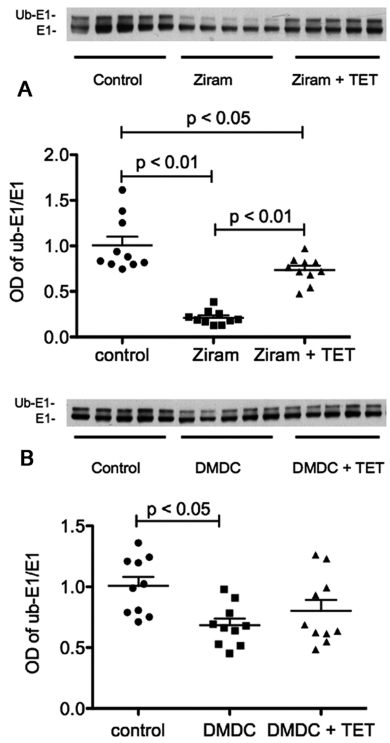


Figure 1. Inhibition of E1 activation by 10 μM ziram (A) and 20 μM DMDC (B) in the presence and absence of 60 μM TET. Representative western blots show activated (ub-E1) and nonactivated (E1) species of E1 ubiquitin activating enzyme separated by SDS-PAGE. Graphs depict the mean ratio of ub-E1/E1 values (+SE) for each treatment group. Statistical comparisons were performed using one-way ANOVA followed by Tukey's multiple comparison test.

DOI: 10.1021/tx500450x
Chem. Res. Toxicol. 2015, 28, 682−690

modification occurred on the reactive E1 cysteines identified for DEDC in vivo, we searched for covalent modifications in E1-enriched HEK293 extracts using LC/MS/MS. On the basis of the structures of ziram and DMDC and the previous adduct identified for DEDC, we analyzed for an *S*-methylaminocarbonyl adduct corresponding to a mass of +58 Da on all peptides identified in the digests (typically, 70−80% sequence coverage was obtained). However, in contrast to our in vivo studies, this adduct was not detected in the cell extracts of the treated HEK293 cells (data not shown). To evaluate the potential of glutathiotinylation, we also searched for +305 Da modifications, but no evidence for this modification was observed. This suggests that significant E1 inhibition was produced by ziram and DMDC either at levels of E1 adduction that were not detectable by LC/MS/MS or through a mechanism independent of cysteine adduction. The shorter exposure duration used in the cell cultures or a difference in the capacity of the HEK293 cells to bioactivate these dithiocarbamates may have contributed to this observation. Additionally, although DMDC and DEDC are structurally very similar and subject to the same metabolic pathways, a smaller contribution from the bioactivation pathway leading to adduct formation in DMDC's biotransformation cannot be ruled out from this study. While these experimental limitations may be responsible for the lack of detectable adducts, the results highlight the possibility that other cellular mechanisms contribute to our observed inhibition of E1. Earlier studies in cell models suggest that E1 activation is redox sensitive, and in vivo studies have demonstrated that dithiocarbamates complex metals and cross cell membranes, potentially increasing intracellular levels of redox active metals.[16,23,24,29] Thus, we reasoned that exposure to ziram and DMDC may be contributing to the observed E1 inhibition by promoting intracellular oxidation, a mechanism that could potentially be shared by many environmental agents and disease conditions. Therefore, our results prompted us to investigate this alternative mechanism.

Previous studies reported E1 activity to be reduced in states of oxidative stress, and certain dithiocarbamates have been shown to complex and transport redox active copper into cells.[22,25,26,30] To explore the possibility that decreased ub-E1 levels in our cell culture assay resulted from an increase in intracellular copper, we exposed cells to 10 μM ziram or 20 μM DMDC in the presence of a known copper chelator, TET, and determined ub-E1 levels (Figure 1). Sixty micromolar TET significantly mitigated the decrease of ub-E1 due to ziram exposure but did not completely prevent decreased ub-E1 levels when coincubated with DMDC. Subsequent studies using coincubation with 120 μM TET did maintain ub-E1 levels similar to that of the control in DMDC-treated samples (data not shown). Thus, we chose to continue our experiments utilizing 60 μM TET, as this was sufficient to maintain ub-E1 levels with ziram treatment and minimize loss of ub-E1 in DMDC-treated samples. We next determined the changes in intracellular levels of copper and zinc, produced by ziram or DMDC, with and without TET. Our results show that copper levels were significantly elevated in cells treated with either dithiocarbamate alone, albeit to different extents (Figure 2). In ziram-treated samples, copper levels were 3-fold higher than that in control, whereas DMDC produced a smaller but significant increase. Zinc measurements revealed an approximately 2-fold increase in intracellular zinc for ziram, with no significant change for DMDC treatment. Thus, although the incubation conditions exposed the cells to molar equivalents of

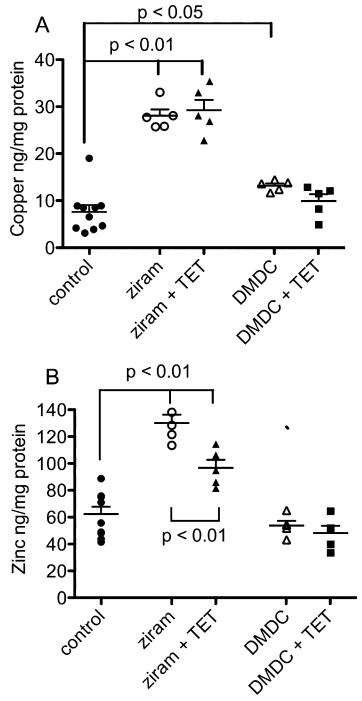


Figure 2. Intracellular zinc and copper levels in samples treated with 10 μM ziram (A) and 20 μM DMDC (B) in the presence and absence of 60 μM TET. Mean values (+SE) are shown. Statistical comparisons were performed using one-way ANOVA followed by Dunnett's multiple comparison test.

N,*N*-dimethyldithiocarbamate, the zinc complexed formulation was more effective in transporting copper and zinc intracellularly and inhibiting E1 than the sodium salt.

Although 60 μM TET prevented intracellular copper transport by DMDC, it had no effect on intracellular copper levels in ziram-treated cells. Zinc also remained significantly elevated in ziram-treated cells. Therefore, it appeared that the ability of TET to decrease ziram-mediated inhibition of E1 was not entirely dependent on preventing intracellular transport of copper or zinc. To address the possibility that TET maintains E1 activity through moderating dithiocarbamate-promoted oxidative injury, we determined the ability of TET to decrease ziram- and DMDC-mediated lipid peroxidation, protein oxidative damage, and expression of heme oxygenase-1 (HO-1). For lipid peroxidation, we utilized a previously established in vitro system composed of an emulsion of ethyl arachidonate incubated with $CuCl_2$ in the presence of either ziram or DMDC, with or without TET, and measured production of MDA.[23] Both ziram and DMDC significantly increased MDA, and similar to intracellular transport of copper, ziram was more effective in generating MDA (Figure 3). Coincubation with TET prevented MDA production for both compounds. To evaluate protein oxidative damage, we measured protein carbonyls in HEK293 cells. Both ziram and DMDC produced a significant increase in the levels of protein carbonyls relative to controls, and TET was effective in preventing the elevated protein carbonylation produced by both dithiocarbamates (Figure 4). Thus, although TET did not completely prevent the intracellular transport of copper, it did diminish the oxidative stress associated with dithiocarbamate exposure, possibly through sequestering copper and reducing the level of dithiocarbamate copper complexes.

DOI: 10.1021/tx500450x
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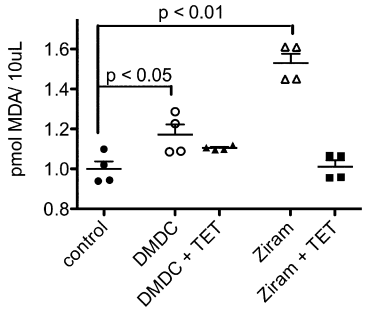

Figure 3. MDA production by ziram and DMDC in the presence and absence of TET. MDA levels were determined in an emulsion of 4 mM ethyl arachidonate, 4 mM ascorbic acid, 100 μM $CuCl_2$ treated with 50 μM ziram, or 100 μM DMDC with and without 600 μM TET as well as in controls incubated similarly without dithiocarbamate or TET. Statistical comparisons were performed using one-way ANOVA followed by Dunnett's multiple comparison test.

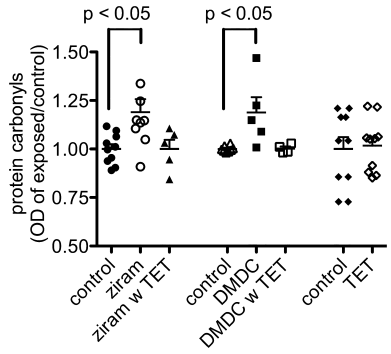

Figure 4. Carbonylated protein determinations in samples treated with 10 μM ziram and 20 μM DMDC in the presence and absence of 60 μM TET. Protein samples were obtained from control cells incubated with DMSO vehicle and exposed cells incubated with either 10 μM ziram or 20 μM DMDC in the presence and absence of 60 μM TET, reacted with 2,4-dinitrophenylhydrazine, and quantified by western blot using an anti 2,4-DNP antibody. Mean optical density (OD) values (+SE), expressed as a ratio to GAPDH as an intra sample standard and then normalized to control mean values, are shown. Statistical comparisons were performed using one-way ANOVA and Dunnett's multiple comparison test or two-way unpaired Student's *t* test (control vs TET).

HO-1 protein expression levels are regulated by the antioxidant response element, have been shown to increase from dithiocarbamate exposure, and can be used as a marker for the cellular response to oxidative stress.[24] Both DMDC and ziram significantly increased HO-1 protein expression (Figure 5). TET prevented the increased HO-1 expression produced by DMDC. However, while the addition of 60 μM TET did not significantly reduce this effect for ziram, coincubation with 120 μM TET did significantly decrease HO-1 levels relative to ziram alone. Because transcriptional regulation of HO-1 includes participation of a metal response element present in the 5′ regulatory region of the gene in addition to the antioxidant response element, the observed HO-1 expression for ziram likely reflects the elevated levels of copper and zinc that remained in the TET and ziram-treated cells.

To delineate the relative contributions of copper and zinc to E1 inhibition, we performed experiments under metal-free media incubation conditions in which copper and zinc levels could be modulated independently. Cells maintained in EMEM were washed with PBS, incubated in Hank's balanced salt

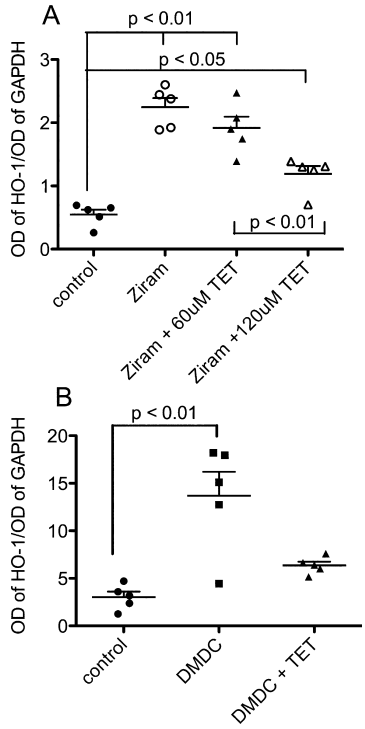

Figure 5. Protein expression levels of heme oxygenase-1 (HO-1) in cells treated with 10 μM ziram (A) and 20 μM DMDC (B) in the presence and absence of TET. The expression level of HO-1 was determined by western blot. Results are shown as the mean (+SE) ratio to GAPDH within the same samples. Statistical comparisons were performed by one-way ANOVA and Tukey's multiple comparison test; $n = 5$ for all groups.

solution (HBSS), and exposed to ziram or DMDC in the presence and absence of added $ZnCl_2$ or $CuCl_2$ and to either $ZnCl_2$ or $CuCl_2$ alone. Under these conditions, all test compounds significantly decreased ub-E1 levels relative to that in controls, and combining DMDC with either $ZnCl_2$ or $CuCl_2$ resulted in a further decrease of ub-E1 relative to that in cells treated with DMDC alone (Figure 6). In the lysates obtained from cells incubated with ziram combined with $CuCl_2$,

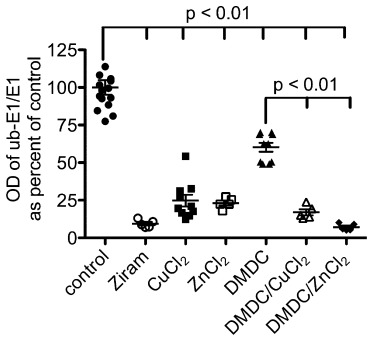

Figure 6. Inhibition of E1 activation in HBSS under metal-free culture conditions. Cells were exposed to 10 μM ziram, 20 μM $CuCl_2$, 20 μM $ZnCl_2$, 20 μM DMDC, 20 μM DMDC plus 20 μM $CuCl_2$, or 20 μM DMDC plus 20 μM $ZnCl_2$. Graphs depict the mean ratio of ub-E1/E1 values (+SE) for each treatment group as the percent of control. Statistical comparisons were performed using one-way ANOVA followed by Tukey's multiple comparison test. In samples incubated with 10 μM ziram plus 20 μM $CuCl_2$, bands were not detectable for either ub-E1 or E1.

DOI: 10.1021/tx500450x
Chem. Res. Toxicol. 2015, 28, 682−690

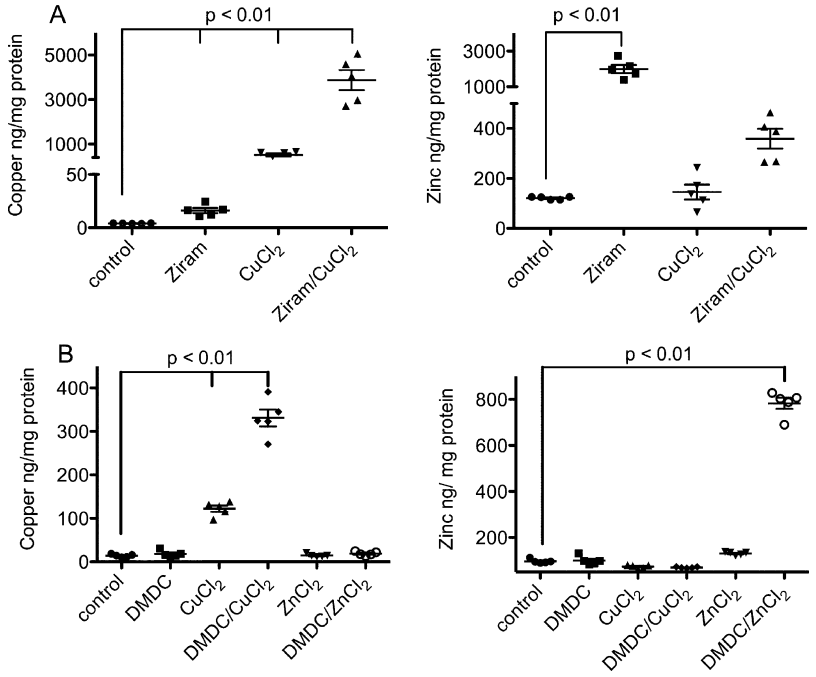

Figure 7. Intracellular copper and zinc levels in HBSS under metal-free culture conditions. Cells were exposed to 10 μM ziram or 20 μM DMDC, 20 μM $CuCl_2$, 20 μM $ZnCl_2$, 10 μM ziram plus 20 μM $CuCl_2$, 20 μM DMDC plus 20 μM $CuCl_2$, or 20 μM DMDC plus 20 μM $ZnCl_2$. Mean values (+SE) are shown. Statistical comparisons were performed using one-way ANOVA followed by Tukey's multiple comparison test.

neither a band for ub-E1 nor E1 could be detected by western blot. Under these metal-free incubation conditions, ziram still significantly increased intracellular zinc and copper, whereas DMDC did not significantly change either metal (Figure 7). The zinc contained in ziram can account for the elevation of zinc in this incubation system, but the ability of ziram to elevate copper demonstrates the greater efficiency of ziram relative to DMDC to either scavenge trace quantities of copper or to mobilize copper located within the cell membrane. The ability of $ZnCl_2$ or $CuCl_2$ alone to inhibit E1 and to enhance the effect of both dithiocarbamates is consistent with the elevation of either zinc or copper contributing to inhibition of E1. Thus, the relative efficacy of ziram and DMDC to elevate intracellular levels of these metals could account for their relative potency for E1 inhibition. However, considering the data obtained from coincubation of the dithiocarbamates with TET, the elevation of zinc or copper alone is not sufficient but also requires that the metals be in a redox-active form accompanied by increased oxidative stress. An unexpected finding was the lack of a significant change in intracellular copper for DMDC even though it produced a significant decrease in ub-E1. Although it cannot be determined from the current study, DMDC may have produced a small but biologically relevant increase in copper that was not detectable in our methodology or it may have shifted a fraction of the protein-associated intracellular copper to a more redox-active form, e.g., a DMDC copper complex.

The studies presented here support intracellular transport of copper and zinc accompanied by oxidative stress as a mechanism for dithiocarbamate-mediated E1 inhibition. These data suggest that E1 is a common target of other environmental toxins that can perturb the redox status of the cell. To further evaluate the potential of our cell culture assay and the use of monitoring E1 function as a generalized response of exposure to environmental toxins, we evaluated ub-E1/E1 ratios in samples treated with a series of compounds, including additional dithiocarbamates, thiocarbamates, triazines, benzimadazoles, and several metals (Table 1). Among the compounds tested, seven of the pesticides produced a significant decrease in ub-E1, and four produced a significant increase in activated E1 when compared to that in the control. We also observed a significant increase in ub-E1 with exposure to $Fe(ClO_4)_2$ and 4-hydroxynonenal as well as a significant decrease in ub-E1 by $MnCl_2$ and the E1 specific inhibitor, pyr-41, compared to that in control samples. It is interesting to note that while the majority of the compounds that altered E1 function did so by inhibiting its activity, some compounds and one metal increased ub-E1. While, to date, our investigations have supported two putative mechanisms by which E1 activity may be altered, the modulation of E1 activity by the compounds in Table 1 suggests that additional mechanisms may be elucidated through continued analysis of altered E1 function.

Collectively, our results are consistent with E1 regulating downstream targets, at least partially, in response to intracellular levels of ROS. Two earlier studies also support this concept.[16,29,31] In those studies, E1 inhibition increased when the antioxidant capacity was diminished in PC12 cells through depletion of glutathione, and upon reestablishment of normal levels of glutathione, E1 activity returned.[29,31] More recent studies in yeast cultures also showed that reactive cysteines on E1 and the E2 conjugase Cdc34 were redox-sensitive and that, when oxidized, they effectively sequestered the proteins' catalytic cysteines, leading to downregulation of cell cycle events dependent on E1 ubiquitination and Cdc34 function.[29] Thus, it is possible that the redox sensitivity of E1 observed in our studies is an integral part of its function and promotes maintenance of cellular homeostasis through its responsiveness to cellular oxidation states.

DOI: 10.1021/tx500450x
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Numerous studies have demonstrated the importance of ubiquitin regulation to the development and function of the central nervous system. The importance of E1 activity in nervous system function is underscored by the fact that mutations in E1 rarely result in viable offspring and infants that survive do so briefly and suffer from X-linked infantile spinal muscular atrophy (XL-SMA).[32] Identifying E1 as being susceptible to perturbation by environmental toxins lends support to the premise established by multiple epidemiological studies that environmental pesticides are potential contributors to the etiology of neurodegenerative disease. In fact, the singular nature and apical positioning of E1 in the cascade of ubiquitin-regulating events make it a strong candidate as a gateway molecule for environmental perturbation of cellular function. Furthermore, E1 susceptibility in concert with increased sensitivity of gene variants of UPS-related proteins, as reported in Rhodes et al., could potentiate or compound the individual contribution of each of these biological factors.[15] The studies reported here support this premise and suggest that the redox-sensitive nature of E1 may be an essential part of its biological function as well as contribute to its vulnerability to deregulation by environmental agents. Continued assessment using both in vivo and in vitro models is essential to further our understanding of how alterations in E1 activity produced by environmental factors can influence biological systems and contribute to neurological disease.

■ ASSOCIATED CONTENT

Supporting Information

Activated E1 levels in control and 1 μM ziram-treated samples. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

This work was supported by the National Institutes of Environmental Health Sciences, grant nos. RO1 ES019969 (to W.M.V.) and P30 ES00267, The Vanderbilt Center in Molecular Toxicology.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We are grateful to Yaofang Zhang and the Mass Spectrometry Research Center Proteomics Laboratory at the Vanderbilt Medical Center for help with measuring the intracellular metal content of our samples, as well as W. Hayes MacDonald from the same facility for help in obtaining the LC/MS/MS data. We also extend our thanks to Kalyani Amaranth for her assistance with the MDA assay.

■ ABBREVIATIONS

UPS, ubiquitin proteasome system; E1, activating enzyme E1; ub-E1, ubiquitinated E1; SN_2, biomolecular nucleophilic substitution; DEDC, *N*,*N*-diethlyldithiocarbamate; PD, Parkinson's disease; DMDC, *N*,*N*-dimethyldithiocarbamate; ATCC, American Type Culture Collection; EMEM, Eagle's minimum essential media; TET, triethylenetetramine hydrochloride; PBS, phosphate buffered saline; PBST, PBS containing 0.05% Tween-20; BSA, bovine serum albumin; HO-1, heme-oxygenase 1; DTT, dithiothreitol; TEA, triethanolamine imidoester; DMP, dimethyl pimelimidate·2HCl; DNPH, 4-dinitrophenylhydrazine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HRP, horseradish peroxidase; MDA, malondialdehyde; BHT, 2,6-di-*tert*-butyl-4-methylphenol; DETBA, diethyl thiobarbiturate acid; TMP, 1,1,3,3-tetramethyoxypropane; ICPMS, inductively coupled plasma mass spectrometry; Me-DETC, *S*-methyl-*N*,*N*-diethylthiocarbamate; Me-DETCSO, *S*-methyl-*N*,*N*-diethylthiocarbamate sulfoxide; EITC, ethylisocyanate; HBSS, Hank's balanced salt solution

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