ZIRAM CAUSES DOPAMINERGIC CELL DAMAGE BY INHIBITING E1 LIGASE OF THE PROTEASOME

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Running head: Ziram Causes Dopaminergic Cell Damage
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The etiology of Parkinson’s disease (PD) is unclear but may involve environmental toxins such as pesticides leading to dysfunction of the ubiquitin proteasome system (UPS). Here, we measured the relative toxicity of ziram (a UPS inhibitor) and analogs to dopaminergic neurons and examined the mechanism of cell death. UPS (26S) activity was measured in cell lines after exposure to ziram and related compounds. Dimethyl- and diethylthiocarbamates including ziram were potent UPS inhibitors. Primary ventral mesencephalic cultures (VMC) were exposed to ziram and cell toxicity was assessed by staining for tyrosine hydroxylase (TH) and NeuN antigen. Ziram caused a preferential damage to TH+ neurons and elevated α-synuclein levels but did not increase aggregate formation. Mechanistically, ziram altered UPS function through interfering with the targeting of substrates by inhibiting ubiquitin E1 ligase. Sodium dimethylthiocarbamate administered to mice for two weeks resulted in persistent motor deficits and a mild reduction in striatal TH staining but no nigral cell loss. These results demonstrate that ziram causes selective dopaminergic cell damage in vitro by inhibiting an important degradative pathway implicated in the etiology of PD. Chronic exposure to widely used dithiocarbamate fungicides may contribute to the development of PD and elucidation of its mechanism would identify a new potential therapeutic target.

Parkinson’s Disease (PD) is a common neurodegenerative disease characterized by relatively selective degeneration of dopaminergic (DA) neurons in the substantia nigra (nigrostriatal neurons). The etiology probably involves both environmental and genetic factors including pesticide exposure (1-3). Hundreds of pesticides are used alone or in combinations making it difficult to separate individual effects. Since no individual pesticide has been established by epidemiologic studies, we chose to perform an unbiased screen of potential toxicants for their ability to interfere with the ubiquitin-proteasome system (UPS), a biological pathway implicated in the etiology of PD. Impaired UPS activity has been reported in brains of patients with PD and mutations in two UPS genes, Parkin and UCH-L1, cause rare genetic forms of PD (4). Although these results are not universally reproduced (5-7), in some studies administration of UPS inhibitors to rodents recapitulates many of the clinical and pathological aspects of PD (8-10). We hypothesized that chronic pesticide exposure may increase the risk of developing PD by inhibiting the UPS. We screened several pesticides for their ability to inhibit the UPS and found a number of toxicants that can lower activity at relevant concentrations (11). We then focused on dithiocarbamate fungicides since they were found to be one of the most potent UPS inhibitors and are widely used in crop protection.

In the present study, zinc dimethylthiocarbamate (ziram) was one of
several dimethyl- and diethylthiocarbamates found to inhibit the UPS at 0.15-1 μM. Furthermore, ziram increased α-synuclein expression in DA cells, induced relatively selective DA cell damage in vitro, and inhibited the UPS by interfering with ubiquitin E1-ligase activity. In vivo, systemic administration of the more soluble sodium dimethylthiocarbamate (NaDMDC) in mice resulted in motor deficits and damage to the nigrostriatal pathway. These findings help understand how chronic pesticide exposure could increase the risk of developing PD.

**EXPERIMENTAL PROCEDURES**

**Chemicals**— The test compounds (Table 1 and Supplement to Table 1) were from Chem Service (West Chester, PA), Sigma-Aldrich (St. Louis, MO) or other commercial sources as the highest available purity except for 7 and 8 which were synthesized by Karl Fisher in the Casida laboratory.

**Measurement of 26S proteasome activity and cell death in cell lines**— 26S and 20S UPS activity and cell death were measured in human embryonic kidney (HEK) and neuroblastoma SK-N-MC cells by flow cytometry as previously described (12). Fluorescence or the green fluorescence protein degron fusion protein (GFP-U) was measured and expressed as percent of control.

**Rat primary ventral mesencephalic cultures (VMC)**— VMC were prepared by using a protocol adapted from Rayport et al. (13). Briefly, the cultures were prepared in two stages. In the first stage, cortical glial feeder cells were established on polyornithine/laminin-coated coverslips, which formed the base of a 10 mm dia. well cut into 35 mm culture dishes, until they reached confluency in approximately 6 days. Fluorodeoxyuridine (FUdR) was then added to prevent additional glial proliferation. In the second stage, postnatal day 2 to 3 pups were anesthetized and 1 mm³ mesencephalic blocks containing the ventral tegmental area were dissected from sagittal sections taken along the midline of the brain. Cells were dissociated and plated onto pre-established glial feeder cells at densities of 1 x 10⁵ per coverslip. The mixed cultures were grown in chemically defined media containing FDU for 10 days before treatment and analysis.

**Immunocytochemistry in VMCs and cell counts**— At the conclusion of the experiment, cultures were immediately washed and fixed in 4% paraformaldehyde for 30 min. The cultures were then incubated with anti-tyrosine hydroxylase (TH) antibodies (1:500, Calbiochem) and anti-NeuN antibodies (1:100, Chemicon) overnight at 4°C. The cells were stained for 2 h with FITC- and Cy3-conjugated secondary antibodies (Jackson Immuno). In some experiments, cells were stained with anti-TH and anti-α-synuclein (1:500, Zymed/Invitrogen) antibodies. After staining and prior to counting, coverslips were randomly assigned an identification number. TH- and NeuN-immunoreactive neuron counts were then determined manually by raters blinded to the experimental conditions. For TH, the entire coverslip was counted but for NeuN+ neurons, representative fields from each coverslip were counted and averaged because of their large number.

**Determination of Dopamine Content in VMCs**— VMCs were homogenized in 0.1 M perchloric acid containing 0.1% EDTA. Insoluble debris was removed by centrifugation, and the supernatant was filtered through a Millipore MC cartridge. The filtrate was analyzed for dopamine by HPLC with electrochemical detection (Antec Leyden, Leiden, The Netherlands) using a mobile phase consisting of sodium acetate (75 mM), sodium dodecane sulfonate (0.75 mM), EDTA (10 mM), triethylamine (0.01%), acetonitrile (12%), methanol (12%), and tetrahydrofuran (1%), pH 5.5, pumped at a rate of 200 μl/min (model LC-10AD; Shimadzu, Columbia, MD) through a 100 x 2 mm column (3 μm, Hypersil C18; Keystone Scientific, Bellefonte, PA). The data were collected and analyzed using ChromPerfect software (Justice Innovations, Mountain View, CA).

**Quantitation of α-synuclein expression**— TH positive cells in each coverslip were identified under 10X, and digital images were obtained using a 40X objective for TH and α-synuclein immunoreactivity. Exposure times were held constant between coverslips. After acquisition, the images were randomly assigned an identification number and the experimental conditions blinded. All images were stacked into a single sequence, a
polyangular region-of-interest (ROI) was manually
drawn around the neuron, and the mean image
intensity, total image intensity, and the area inside
the ROI were evaluated for each cell. Data
analysis was performed with IPLab ver 3.x for
Windows (Scalytics Inc.) compensating for
bleed through between FITC and Cy3 channels.

In order to determine if ziram treatment
leads to high molecular weight (HMW) α-
synuclein species, we performed Western blots on
the detergent-soluble fractions of culture lysates.
VMCs treated with either 1 μM ziram or 5 μM
lactocystin were lysed in buffer containing 1%
Triton X-100 and 0.1% SDS. Lysates were
sonicated and insoluble debris was removed by
centrifugation. Protein concentrations were
determined and 10 mg/lane were subjected to
SDS-PAGE. The separated proteins were
transferred to nitrocellulose membranes and
incubated with an anti-α-synuclein antibody (BD
Transduction Laboratories, 1:10000) followed by an
anti-tubulin antibody for normalization.

Evaluation of E1 ligase activity- Ziram’s effects
on E1 ligase activity were investigated using
Western blot analysis of treated cellular extracts to
determine E1/E1-ubiquitin ratios and using
purified enzyme preparations. Ziram treated SK-
N-MC cells were washed with PBS then lysed in a
thiol stabilizing buffer using the method of Jha et
al.(14). The samples were sonicated for 10 s,
centrifuged for 15 min at 13,000 g, mixed with 2
parts thiol gel buffer (33 mM Tris-HCl, pH 6.8,
2.7 M urea, 2.5% sodium dodecyl sulfate and 13
% glycerol), boiled for 2 min and 10 μg protein
per lane was loaded on a 12% SDS-PAGE gel(15).
Proteins were electrophoretically transblotted onto
nitrocellulose paper and immunoblots (IB) were
performed as previously described(16) using anti-
E1 ligase antibody (BIOMICOL, Plymouth Meeting,
PA). Antigen-antibody interactions for IB were
visualized using horse radish peroxidase (HRP)-
conjugated secondary antibody and chemoluminescence substrate (Pierce, Rockford,
IL).

For purified enzyme assays, human
recombinant ubiquitin-activating E1 enzyme and
biotinylated ubiquitin (both from BIOMICOL) were
incubated for 5 or 10 min in thioester buffer per
manufacturer’s protocol. Reactions were stopped
using thiol-stabilizing buffer and proteins were
subjected to SDS-PAGE and transblotted onto
nitrocellulose paper. E1-ubiquitin conjugates
were determined using strepavidin-HRP and an
enhanced chemiluminescence kit. Band densities
were measured using a scanning densitometer.

Animal studies

Male C57BL/6 mice were treated for two
weeks by subcutaneous osmotic minipumps
with NaDMDC (50 mg/kg/day) in PBS or with PBS
only. Behavior of the mice was evaluated using
the pole test as described by Fleming et al except
that a cut off time of 60 sec was used (17). One
week after the last behavioral testing, mice were
perfused with fixative, and their brain sectioned
for TH staining. Fiber density was measured with
a computer assisted image analysis system as
previously described (18).

For stereological analysis in the substantia
nigra pars compacta (SNC), neurons were counted
using the optical fractionator method with the
Stereo Investigator software (Microbrightfield,
Colchester, VT) coupled to a Leica DM-LB
microscope with a Luddi XYZ motorized stage and
z-axis microcator (MT12, Heidenheim, Traunreut,
Germany). The SNC was delineated at 5X
objective using previously reported criteria
(19,20). After delineation at low magnification,
every fourth section throughout the SNC was
counted at 100X magnification.

Statistical analysis- Statistical analysis was
performed with GraphPad Prism software.
Statistical significance was determined using one-
way ANOVA with Dunnett’s Multiple
Comparison Post-Test and Bonferroni’s Multiple
Comparison Post-Test as appropriate. For
histochemistry and behavior, statistical analysis was
performed with GB-Stat software (Dynamic
Microsystems, Inc. Silver Spring, MD, 2000) for
Macintosh. A 2 x 3 mixed design ANOVA was
followed by post hoc analysis with Fisher’s LSD. In
order to maintain homogeneity of variance, an inverse
transform was calculated (21) for each score in the pole
test. The level of significance was set at p<0.05.

RESULTS

Ziram inhibits 26S proteasome activity in HEK
cells- We reported earlier that ziram inhibits 26S
UPS in SK-N-MC cells expressing a GFP-degron
reporter system(11). In order to determine if
ziram causes a similar inhibition in a non-neuronal
cell line, HEK cells were exposed and GFP accumulation was measured using flow cytometry. We found that HEK cells were similarly sensitive to the 26S inhibitory effects of ziram with an IC50 of 161 nM (Figure 1).

**Structure-activity relationships of ziram-related compounds for UPS inhibition**

Ziram and 14 related compounds were tested at 1 and 10 μM for their ability to inhibit 26S UPS activity in GFP1-transfected SK-N-MC cells (Table 1). Ziram (1) and the other dimethylthiocarbamate (DMDC) derivatives [sodium DMDC (2), free acid (3), and bis-disulfide (4)] were similarly very active. Two diethylthiocarbamates (5 and 6) were also very potent. Thiram (4) and disulfiram (6) are cleaved into DMDC (3) and diethylthiocarbamate (5), respectively. Ziram in aqueous solution dissociates to zinc ion (7) and forms dithio acid 3 which breaks down into carbon disulfide (8) and dimethylamine (9) all of which were inactive. The disulfide functionality of etem (10) [an ethylenebis(dithiocarbamate) fungicide oxidation product can cleave to a dithiocarbamic acid possibly associated with its activity whereas that of the phenyl analog (11) cannot give a dithiocarbamate. A carbon disulfide progenitor, enzone (12), was also inactive. Environmental degradation converts ethylenebis(dithiocarbamate) fungicides to ethylenethiourea (13) and sodium methylthiocarbamate (14) to methyl isothiocyanate (15), all of which were inactive. Thus, the dialkylthiocarbamate moiety is required to alter UPS activity. Furthermore, pre-incubation of ziram with the reducing agents glutathione (GSH), DTT, or N-acetylcysteine, completely abolished its inhibitory activity (data not shown).

**Ziram causes dopaminergic cell damage in primary VMC**

Since proteasome inhibition has been implicated in the etiology of PD and ziram causes UPS dysfunction, dopaminergic cells might be selectively vulnerable to ziram’s toxicity. Primary VMC were exposed to ziram for 48 h then cellular toxicity was measured by counting TH immuno-positive (TH+) cells and NeuN immuno-positive (NeuN+) cells (a non-specific neuronal marker). Ziram at 5 and 10 μM was highly toxic to all cells, including the glial bed, causing the cell layers to lift off the coverslip after treatment or during immunostaining (data not shown). At lower doses Ziram exposure had a significant overall effect on TH+ cell survival (F4,110 = 4.52, p<0.005) reducing TH+ cell number at 0.5 μM and 1 μM (p<0.05, Dunnet’s post-hoc test versus control; Figure 2). The small, non-significant decrease in total NeuN+ cells measured after ziram treatment therefore represents primarily the loss of the TH+ subset of the entire NeuN+ pool. The loss of TH+ cells is likely reflective of cell death and not simply loss of expression because TH levels were increased in the surviving positive cells (data not shown).

The 20S proteasome inhibitor lactacystin is reported to cause selective DA cell death in VMC but this has not been a universal finding [Rideout, 2005 #1361; Kikuchi, 2003 #1362; Hoglinger, 2003 #1363; McNaught, 2002 #1256; Petrucelli, 2002 #1364]. In the present study lactacystin was toxic to NeuN+ neurons (F4,110 = 13.12, p<0.0001) and to the TH+ subset of such neurons (F4,110 = 4.26, p<0.003) but the TH+/NeuN+ ratios revealed that the effects of lactacystin were not specific to dopaminergic neurons (F4,110 = 0.90, p>0.05, Figure 2). Since ziram caused preferential loss of TH+ neurons and lactacystin did not, they appear to act via different mechanisms despite the fact that they are both UPS inhibitors.

**Ziram toxicity is not dopamine dependent**

One possible mechanism for the relative selective effect to TH+ neurons is that ziram interacts with dopamine metabolism to produce preferential toxicity. To test this hypothesis, ziram toxicity was measured in the presence of α-methyl L-tyrosine (α-MT), an inhibitor of dopamine synthesis. Treatment of VMCs with α-MT (250 μM) for 48 h resulted in a decrease in dopamine content of 62 ± 6% compared to controls (p ≤ 0.05) but did not significantly alter the number of TH+ cells. Reducing dopamine content with α-MT was ineffective in attenuating ziram’s toxicity to TH+ neurons (Figure 3).

**α-Synuclein expression in VMCs**

Several converging lines of evidence support a role for α-synuclein in the pathogenesis of PD. Mutations in
the α-synuclein gene or increased expression of wild-type α-synuclein cause PD in familial PD and α-synuclein is a major component of Lewy Bodies in sporadic PD (22). In order to determine if ziram alters the levels of α-synuclein in VMCs, neurons were stained for both TH and α-synuclein and relative fluorescence was measured. TH+ neurons showed robust and punctate α-synuclein staining, suggesting that they are mature and terminally differentiated (23,24). Ziram at 0.5 μM resulted in increased α-synuclein expression while lactacystin at 5 μM decreased expression (Figure 4). The sizes of the ROIs were the same between all conditions varied in all experiments (data not shown).

An important pathological marker in PD is the formation of α-synuclein inclusions or aggregates. TH+ cells from ziram and lactacystin treated VMCs were determined to be either positive or negative for nuclear, peri-nuclear, or cytoplasmic α-synuclein inclusions by blinded raters. Aggregates were relatively common in both treated and untreated cells but surprisingly no significant differences were found with ziram compared to controls. Interestingly, a decrease in nuclear and peri-nuclear aggregates was seen in the lactacystin-treated cultures (Table 2).

In order to determine if ziram treatment results in increased formation of detergent-soluble α-synuclein oligomer formation, we subjected VMCs lysates to Western blot analysis. Both monomeric and oligomeric forms of α-synuclein were apparent in detergent-soluble fractions as previously described (25). Ziram treatment resulted in a non-significant trend for an increase in oligomeric forms of α-synuclein compared to controls (170 ± 120% optical density units of controls, N=8 for ziram and N=5 for controls, P=0.18). Oligomeric α-synuclein was unchanged in lactacystin-treated VMCs and monomeric α-synuclein levels were similar in all 3 conditions (data not shown).

**Ziram inhibits E1 ligase activity** - Ziram was found to inhibit the UPS using an assay that requires the substrate (i.e. degron) to be ubiquinylated via ubiquitin ligases and recognized by the 26S proteasome before it can be degraded by 20S proteases (12). Disruption of any of these steps would be detected in the screen. It has been suggested that another dithiocarbamate fungicide, maneb, inhibits the 20S component of the UPS (26). In order to determine if ziram acted in this manner, HEK cells were treated with ziram (1 and 10 μM) for 24 h but there was no change in 20S chymotryptic activity (data not shown). The amount of α-subunit of the 20S proteasome was also measured using Western blot analysis and no differences were found (data not shown). Furthermore, our earlier study showed that ziram had no effect on 20S UPS activity when added directly to cell lysate (11). Since 20S proteolytic activity was not altered by ziram, Western blot analysis on lysates was performed from ziram treated cells to determine if ubiquitinated proteins accumulated. As expected, inhibition of the 20S UPS by lactacystin and rotenone resulted in accumulation of high molecular weight ubiquitin conjugates (Figure 5). Conversely, ziram treatment resulted in a significant decrease in these proteins suggesting that it inhibits the UPS by interfering with ubiquitin ligation. Polyubiquitin is added to proteins targeted for UPS degradation by a series of ligases (E1, E2, and E3 ligases). Dithiocarbamates can lead to GSH oxidation (27) and GSH depletion results in loss of E1 ligase activity (14). Ziram does not appear to act by lowering GSH since depleting cellular GSH using buthionine sulfoximine (1 mM) had no effect on UPS activity and ziram did not alter the amount of reduced GSH in the cells at concentrations up to 50 μM (data not shown). Ubiquitin is activated by E1 ligase in an ATP-dependent manner forming an E1-ubiquitin adduct before the ubiquitin is transferred to E2 ligase. Since activated ubiquitin binds covalently to a cysteine of E1, ziram’s thiol group might interfere with this reaction. Indeed, ziram markedly reduced E1 ligase activity as measured by a reduction of endogenous E1-ubiquitin conjugates and the reduced formation of E1-ubiquitin conjugates in a purified preparation (Figure 6).

**Motor deficits in NaDMDC-treated mice** - NaDMDC was used for in vivo experiments because of its greater solubility than the zinc DMDC salt, ziram, with similar effect on proteasomal function (Table 1). Treatment of male C57BL/6 mice for two weeks with NaDMDC resulted in increased time to turn in the pole test, a deficit also seen in mice with dysfunction of the nigrostriatal pathway (Figure 7). The effect

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persisted 8 weeks after cessation of drug treatment, indicating that it was not due to an acute drug effect. Striatal TH fiber density was non-significantly reduced across striatal regions at 2 weeks, with a significant reduction in the ventrolateral quadrant 9 weeks after drug treatment in the NaDMDC-treated animals (Table 3). Stereological TH immunoreactive neuron counts in the SNc of treated mice were not changed (Table 3). Thus, 2 weeks treatment of mice with NaDMDC, an E1 activating enzyme inhibitor, resulted in motor deficit with minor loss of nigrostriatal dopaminergic fibers

**DISCUSSION**

**Pesticides, Ziram and PD-** The most consistent and strongest association between a group of environmental toxicants and the development of sporadic PD has been with chronic pesticide exposure although no specific agents have been identified (3). Preliminary data from our population-based study in Central California that determined pesticide exposure using a state application registry has revealed some intriguing results. Subjects living within 500 meters of where ziram was applied were at an over 3 fold higher risk of developing PD compared to those with lower exposure (Ritz B, Bronstein JM, Costello S., unpublished data). Chronic inhibition of the UPS has been implicated in the pathogenesis of PD and some pesticides might increase the risk of developing PD by causing UPS dysfunction. The widely-used pesticide ziram is one of the most potent inhibitors of 26S UPS (11). This study further demonstrates that ziram kills TH+ cells in a relatively selective manner, increases α-synuclein levels, and inhibits E1 ligase activity thus interfering with the targeting of proteins destined for UPS degradation. If these preliminary epidemiology findings are confirmed, and taken together with the results of this study, chronic ziram exposure would be a strong candidate PD-associated toxicant. The results presented here add a biologically-plausible mechanism (at relevant concentrations) by which ziram may increase ones risk of developing the disease.

**Mechanism of Ziram Effect on the UPS-** Uncovering how ziram causes UPS dysfunction and cell death might provide important clues to the selective vulnerability of DA neurons. Some of the results in primary cultures were surprising. Although ziram damaged (and probably killed) TH+ cells in a selective manner, this was not the case for the 20S proteasome inhibitor lactacystin. Even though TH staining was used as a marker of DA cell survival, it is possible that the cells were still alive but simply lost their TH phenotype. However, this is unlikely since TH levels were actually increased in the remaining cells after ziram exposure (but not for lactacystin). Conflicting results on proteasome inhibitor-induced selective DA cell death may be due to differences in culturing techniques and conditions (28-32). Cultures in the current study contained DA neurons of the ventral tegmental region (VTA) in order to increase the number of TH+ cells per well and power analysis. Since VTA neurons are believed to be more resistant to stressors, the results likely underestimate ziram’s effects on SNc DA neurons. Indeed, pilot experiments using predominantly nigral cultures yielded similar results. The fact that different effects were found with ziram compared to lactacystin suggests that they act through different mechanisms. As opposed to lactacystin, a 20S protease inhibitor, we show that ziram acts upstream by interfering with ubiquitin ligation. Since ubiquitylation is also important in many cellular processes in addition to the UPS, including modification of protein function, facilitation of cell-surface-receptor turnover and control of gene transcription, it is possible that some of ziram’s actions may be via alternative pathways (33).

The molecular basis of ziram’s ability to inhibit E1 ligase activity was studied by determining the relative potencies of several of its analogues. The most potent 26S UPS inhibitors were dimethyl- and diethylthiocarbamates and their salts and disulfides. These compounds may act by copper or iron chelation (34) or undergo oxidative activation to the S-oxides of the dithiocarbamic acids (35) or of the S-methyl thiocarbamates (36) which are reactive with GSH and potentially with a thiol group of the UPS E1 ligase (Figure 8).

**Maneb and Ethylenebisdithiocarbamate Fungicides-** The effect of ziram on UPS activity has not been observed by others but the structurally-related fungicide maneb is reported to inhibit 20S protease activity (26). Although ziram
and maneb have many structural features in common, our results with ziram differ significantly from those of Zhou et al. using maneb. In our studies, ziram did not inhibit the 20S proteasome at concentrations up to 10 μM, did not cause oxidative stress as measured by 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCF) fluorescence (11), nor did it induce α-synuclein aggregates in primary cultures. These observations may be due to differences in the compounds and/or cell assays used. Maneb is an ethylenbis(dithiocarbamate) and contains manganese, which possibly could contribute to some of its effects but manganese by itself did not alter 26S UPS activity (data not shown).

Another difference between maneb’s and ziram’s effects is that maneb is reported to induce α-synuclein aggregates in MES cells (rat embryonic mesencephalon murine neuroblastoma-glioma hybrid cell line) (26). The present study did not find an increase in aggregate formation in primary mesencephalic cultures but did not evaluate aggregates in immortalized cell lines because neither HEK or SK-N-MC cells expressed significant levels. Several lines of evidence support a mechanism for ziram upstream of the 20S; inhibition of the formation of high molecular weight ubiquitin conjugates, reduction of the E1-ubiquitin/E1 ratio, and direct inhibition of purified E1 ligase activity. Ziram effects on E2 or E3 ligase activities were not studied since they are dependent on E1 ligase, but since they transfer ubiquitin in a similar manner as E1, it is possible that ziram would have similar inhibitory effects on these enzymes.

Animal Studies on Dithiocarbamate Fungicides

Further support for the potential role dithiocarbamates in PD comes from animal studies. Chronic maneb exposure given with paraquat recapitulates many of the behavioral and pathological hallmarks of PD (37). Two week subcutaneous minipump treatment with NaDMDC at 50 mg/kg/day resulted in persistent motor abnormalities typically seen in mice with dysfunction of the nigrostriatal pathway (38). Abnormal motor behavior in our study was associated with a mild, delayed dopamine nerve terminal damage in the ventrolateral region of the striatum but not with nigral cell death. Motor dysfunction without dopamine cell loss may indicate functional damage to these neurons but we cannot exclude that the behavioral tests were detecting abnormalities in other areas involved in motor control.

In summary, we show that ziram and structurally related dithiocarbamates induce dysfunction of the UPS by inhibiting E1 ligase. We demonstrate that ziram exposure increases α-synuclein levels and selectively damages dopaminergic neurons in primary cultures. Furthermore, chronic system exposure to NaDMDC causes persistent motor deficits and mild injury to the nigrostriatal pathway. This study along with emerging epidemiological investigations suggests that chronic exposure to dithiocarbamate fungicides can contribute to the pathogenesis of PD.

REFERENCES


FOOTNOTES
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FIGURE LEGENDS

Figure 1. Ziram inhibits UPS in HEK cells with 24 h incubation prior to analysis by flow cytometry. The IC50 for ziram was 161 nM.

Figure 2. Ziram- and lactacystin-induced tyrosine hydroxylase cell damage in primary mesencephalic cultures. TH+ neurons were selectively vulnerable to ziram- but not lactacystin-induced damage. Below: Representative photomicrograph of TH- (red) and NeuN- (green) stained mesencephalic cultures. A) control. B) Ziram (1 μM).

Figure 3. Inhibition of dopamine synthesis by α-methyl-L-tyrosine did not attenuate ziram-induced dopamine cell death (n=14-44 wells per condition). * p ≤ 0.05, ziram vs DMSO control. + Not significant.

Figure 4. Ziram increases and lactacystin decreases α-synuclein levels in TH+ neurons. Cells were exposed for 48 h prior to analysis. Relative intensities were measured in a blinded manner in cells that were selected randomly (n=21-97 cells per condition). Representative α-synuclein stained cells are shown on the right. Scale bars=10 microns. ** p ≤ 0.01.

Figure 5. Anti-ubiquitin Western Blot analysis of lysates from cells following treatment with 5 μM lactacystin, 0.1 μM rotenone, or 1 μM ziram. Relative densities were determined using the NIH Image program, normalized to tubulin immunoactivity in the same blot, and expressed as % of untreated controls (n=4). 20S inhibition using lactacystin resulted in an increase in high molecular weight (HMW) ubiquitin conjugates while ziram treatment reduced them. ** p ≤ 0.01, * p ≤ 0.05.

Figure 6. Effect of ziram on E1-ubiquitin conjugates and E1 ligase activity. A. Endogenous E1-ubiquitin conjugates in lysates of ziram-treated HEK cells. Ziram resulted in a dose dependent reduction
in the E1-Ub/E1 ratio compared to untreated controls. Western blot is shown in the insert. B. E1 ligase activity was inhibited by ziram in purified preparations after 5 and 10 min incubation. n=4, p ≤ 0.05.

**Figure 7.** Motor deficits in mice determined by the pole test following 2 weeks subcutaneous minipump treatment with NaDMDC at 50 mg/kg/day followed by 8 weeks post-treatment period (10 weeks from the beginning of the experiment). Inverse Transform: Reciprocal of the original data point. Baseline=1 because untreated mice turn in less than one second. ** p< 0.01 compared to saline treated mice at the same time point. ΔΔ represents p< 0.01 compared to baseline within the same treatment group. 2 x 3 Mixed design ANOVA, Fisher’s LSD.

**Figure 8.** Alternative mechanisms for ziram oxidative activation and reaction with 26S E1 ligase or GSH.
Table 1. Structure-activity relationships for ziram-related compounds on 26S UPS with 24 hour treatment of SK-N-MC cells

<table>
<thead>
<tr>
<th>Compound</th>
<th>Structure(^a)</th>
<th>Fluorescence as percent of control (mean ± SD, n=4)(^b)</th>
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<tr>
<td></td>
<td>Name</td>
<td>1 uM</td>
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<tr>
<td>Dimethyldithiocarbamates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Ziram</td>
<td>([(\text{CH}_3)_2\text{NC(S)S}]_2\text{Zn})</td>
<td>274 ± 21**</td>
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<tr>
<td>2 Sodium dimethyldithiocarbamate</td>
<td>((\text{CH}_3)_2\text{NC(S)SNa}\cdot\text{H}_2\text{O})</td>
<td>200 ± 47*</td>
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<tr>
<td>3 Dimethyldithiocarbamate</td>
<td>((\text{CH}_3)_2\text{NC(S)SH}\cdot\text{H}_2\text{O})</td>
<td>195 ± 9**</td>
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<tr>
<td>4 Thiram</td>
<td>([(\text{CH}_3)_2\text{NC(S)S}]_2)</td>
<td>303 ± 34**</td>
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<td>Diethyldithiocarbamates</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 Diethyldithiocarbamate</td>
<td>((\text{C}_2\text{H}_5)_2\text{NC(S)SH})</td>
<td>167 ± 24*</td>
</tr>
<tr>
<td>6 Disulfiram</td>
<td>([(\text{C}_2\text{H}_5)_2\text{NC(S)S}]_2)</td>
<td>258 ± 25**</td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7 Zinc chloride</td>
<td>\text{ZnCl}_2</td>
<td>95 ± 3</td>
</tr>
<tr>
<td>8 Carbon disulfide</td>
<td>\text{CS}_2</td>
<td>108 ± 8</td>
</tr>
<tr>
<td>9 Dimethylamine hydrochloride</td>
<td>((\text{CH}_3)_2\text{NH\cdot HCl})</td>
<td>91 ± 4</td>
</tr>
<tr>
<td>10 Etem</td>
<td>heterocyclic(^a)</td>
<td>98 ± 5</td>
</tr>
<tr>
<td>11 3H-1,2-Benzodithiole-3-thione</td>
<td>heterocyclic(^a)</td>
<td>95 ± 1</td>
</tr>
<tr>
<td>12 Enzone or GY-81</td>
<td>\text{NaSC(S)SSNa}(^a)</td>
<td>91 ± 4</td>
</tr>
<tr>
<td>13 Ethylenethiourea</td>
<td>heterocyclic(^a)</td>
<td>89 ± 3</td>
</tr>
<tr>
<td>14 Sodium methylthiodithiocarbamate</td>
<td>\text{CH}_3\text{NH(C(S)SSNa})</td>
<td>101 ± 6</td>
</tr>
<tr>
<td>15 Methyl isothiocyanate</td>
<td>\text{CH}_3\text{N=C=S})</td>
<td>91 ± 1</td>
</tr>
</tbody>
</table>

\(^a\)compound 10 is an oxidation product of maneb and other ethylenebis(dithiocarbamate) fungicides; 11 is an analog of 10 with phenyl instead of dihydroimidazole moiety; 12 decomposes readily to \text{CS}_2; 13 is a degradation product of ethylenebis(dithiocarbamate) fungicides.

\(^b\)Higher number reflects UPS inhibition * p<0.05; ** p< 0.01
Table 2. Ziram does not induce the formation of α-synuclein aggregates

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% cells with inclusions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nuclear</td>
</tr>
<tr>
<td>Control</td>
<td>32 ± 5</td>
</tr>
<tr>
<td>Ziram 0.5 µM</td>
<td>38 ± 6</td>
</tr>
<tr>
<td>Ziram 1.0 µM</td>
<td>19 ± 9</td>
</tr>
<tr>
<td>Lactacystin 5 uM</td>
<td>19 ± 4*</td>
</tr>
</tbody>
</table>

n=21-97 cells per condition. * p =0.04, ** p =0.01
Table 3. Effect of subcutaneous treatment with NaDMDC on TH immunoreactivity (IR) fiber density in striatal subregions and stereological estimates of TH-IR neurons in the SNC.

<table>
<thead>
<tr>
<th>Region</th>
<th>Mean ± SEM (n=5)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2W</td>
<td>2 + 9W</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>control</td>
<td>treated</td>
<td>control</td>
<td>treated</td>
</tr>
<tr>
<td>TH-IR fiber density</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DL striatum</td>
<td>1515 ± 207</td>
<td>1456 ± 221</td>
<td>1934 ± 299</td>
<td>1951 ± 269</td>
</tr>
<tr>
<td>DM striatum</td>
<td>1048 ± 149</td>
<td>843 ± 117</td>
<td>1435 ± 199</td>
<td>1358 ± 130</td>
</tr>
<tr>
<td>VL striatum</td>
<td>1439 ± 142</td>
<td>1290 ± 140</td>
<td>1833 ± 254</td>
<td>1409 ± 166*</td>
</tr>
<tr>
<td>VM striatum</td>
<td>815 ± 103</td>
<td>696 ± 62</td>
<td>1073 ± 174</td>
<td>946 ± 152</td>
</tr>
<tr>
<td>SNC</td>
<td>7980 ± 491</td>
<td>7832 ± 574</td>
<td>8251 ± 214</td>
<td>7897 ± 247</td>
</tr>
</tbody>
</table>

DL; dorsolateral; DM; dorsomedial; VL; ventrolateral; VM; ventromedial. SNC: substantial nigra pars compacta. * p ≤ 0.05 compared to corresponding region in control mice at the same time point; ANOVA followed by Fisher PLSD.
Figure 1

![Graph showing the relationship between Ziram concentration (nM) and GFP-U (% control). The graph displays a dose-response curve with increasing GFP-U values as Ziram concentration increases.]
## Figure 2

<table>
<thead>
<tr>
<th>Compound (μM)</th>
<th>TH+ (mean ± SD)</th>
<th>NeuN+ (mean ± SD)</th>
<th>TH+/NeuN+ (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ziram</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>100 ± 7</td>
<td>100 ± 4</td>
<td>1.00 ± 0.05</td>
</tr>
<tr>
<td>0.1</td>
<td>111 ± 22</td>
<td>108 ± 15</td>
<td>0.93 ± 0.11</td>
</tr>
<tr>
<td>0.25</td>
<td>76 ± 7</td>
<td>81 ± 5</td>
<td>1.00 ± 0.07</td>
</tr>
<tr>
<td>0.5</td>
<td>60 ± 10*</td>
<td>75 ± 11</td>
<td>0.78 ± 0.09*</td>
</tr>
<tr>
<td>1</td>
<td>59 ± 10*</td>
<td>81 ± 11</td>
<td>0.64 ± 0.07**</td>
</tr>
<tr>
<td>Lactacystin</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>100 ± 7</td>
<td>100 ± 4</td>
<td>1.00 ± 0.05</td>
</tr>
<tr>
<td>1</td>
<td>89 ± 7</td>
<td>84 ± 4</td>
<td>1.18 ± 0.10</td>
</tr>
<tr>
<td>2</td>
<td>84 ± 11</td>
<td>81 ± 6</td>
<td>1.12 ± 0.14</td>
</tr>
<tr>
<td>5</td>
<td>79 ± 15</td>
<td>71 ± 6**</td>
<td>1.19 ± 0.24</td>
</tr>
<tr>
<td>10</td>
<td>54 ± 6**</td>
<td>50 ± 7**</td>
<td>1.24 ± 0.11</td>
</tr>
</tbody>
</table>

* p < 0.05 ** p < 0.01

---

**A** and **B** indicate the different conditions or treatments.
Figure 3

[Graph showing TH+ cell number percentage control for different concentrations of α-MT (0, 100, 250 μM) and DMSO vs Ziram 0.5 μM.]

- DMSO
  - 0 μM: 100% control
  - 100 μM: 100% control
  - 250 μM: 100% control

- Ziram 0.5 μM
  - 0 μM: 100% control
  - 100 μM: 75% control
  - 250 μM: 50% control

* indicates a significant difference.
**Figure 4**

<table>
<thead>
<tr>
<th>Treatment (μM)</th>
<th>Relative expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.00 ± 0.06</td>
</tr>
<tr>
<td>Ziram 0.5</td>
<td>1.07 ± 0.08</td>
</tr>
<tr>
<td>Ziram 1.0</td>
<td>1.34 ± 0.12*</td>
</tr>
<tr>
<td>Lactacystin 5</td>
<td>0.70 ± 0.07*</td>
</tr>
</tbody>
</table>

* indicates statistical significance.
Figure 5

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mean ± SEM</th>
<th>% Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.46 ± 1.2</td>
<td>100</td>
</tr>
<tr>
<td>Lactocystin</td>
<td>21.41 ± 7.0</td>
<td>618 **</td>
</tr>
<tr>
<td>Ziram (1 μM)</td>
<td>2.44 ± 0.9</td>
<td>71 *</td>
</tr>
</tbody>
</table>
Figure 6

(A) Graph showing E1-Ub/E1 levels at different Ziram Concentrations (nM).

(B) Graph showing Density (ziram/control) at Ziram concentrations of 0.5 µM and 1.0 µM.
Figure 7
Figure 8

\[
\begin{align*}
[(\text{CH}_3)_2\text{NCS}]_2\text{Zn} & \quad \xrightarrow{3\text{ steps}} \quad \text{HS-E1 ligase or GSH} \\
(\text{CH}_3)_2\text{NCSCH}_3 & \quad \xrightarrow{\text{HS-E1 ligase}} \quad (\text{CH}_3)_2\text{NCSG} \\
(\text{CH}_3)_2\text{NCSH} & \quad \xrightarrow{[O]} \quad (\text{CH}_3)_2\text{NCSS-H} \\
(\text{CH}_3)_2\text{NCSS-H} & \quad \xrightarrow{\text{HS-E1 ligase}} \quad (\text{CH}_3)_2\text{NCSSG}
\end{align*}
\]