



Short Communication

Thiol-reactivity of the fungicide maneb

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ABSTRACT

Maneb (MB) is a manganese-containing ethylene bis-dithiocarbamate fungicide that is implicated as an environmental risk factor for Parkinson's disease, especially in combination with paraquat (PQ). Dithiocarbamates inhibit aldehyde dehydrogenases, but the relationship of this to the combined toxicity of MB + PQ is unclear because PQ is an oxidant and MB activates Nrf2 and increases cellular GSH without apparent oxidative stress. The present research investigated the direct reactivity of MB with protein thiols using recombinant thioredoxin-1 (Trx1) as a model protein. The results show that MB causes stoichiometric loss of protein thiols, reversibly dimerizes the protein and inhibits its enzymatic activity. MB reacted at similar rates with low-molecular weight, thiol-containing chemicals. Together, the data suggest that MB can potentiate neurotoxicity of multiple agents by disrupting protein thiol functions in a manner analogous to that caused by oxidative stress, but without GSH depletion.

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Introduction

Maneb (MB), a manganese-containing dithiocarbamate fungicide, is used in agriculture to treat a variety of crop pathologies. Epidemiological studies associate repeated human exposure to agricultural chemicals with increased risk for Parkinson's disease (PD) [1,2] and recent reports indicate that occupational and/or residential exposure to MB significantly increases the risk of developing PD [3–5]. Rodent models of pesticide-mediated PD show that combined effects of paraquat (PQ) and MB exposure leads to deficits in motor function, loss of tyrosine hydroxylase staining in the substantia nigra, and altered striatal dopamine metabolism [6,7]. Mechanistic studies showed that the increased toxicity of PQ + MB could be due, at least in part, to altered toxicokinetics of PQ in mice exposed to MB [8].

In a recent *in vitro* study, we obtained evidence that MB and PQ operate through divergent mechanisms of toxicity [9]. Results showed that PQ acted via a mechanism involving reactive oxygen species (ROS) while MB did not. MB did not increase ROS production or oxidize thiol-containing antioxidants (thioredoxin and peroxiredoxin), but did activate Nrf2. Hong et al. have reported a strong correlation between alkylation of critical Cys residues on Keap-1 and the potency of Nrf2 activation [10]. This report and our data lead to the speculation that MB could be a direct thiol-modifying agent causing Nrf2-Keap-1 dissociation, nuclear translocation and gene transcription. Currently, there is no explicit evidence demonstrating that MB modifies Cys

residues, e.g. Keap-1. Therefore, the purpose of this study was to characterize the thiol binding activity of MB utilizing N-acetylcysteine (NAC) and thioredoxin-1 (Trx1) as model thiol containing agents.

Materials and methods

Materials

Recombinant human thioredoxin-1 protein was obtained from Lab Frontier (Korea). All other reagents were obtained from Sigma.

Reactivity of MB with free thiols

To investigate thiol reactivity of MB, we incubated MB (0–100 μ M) with 50 μ M NAC for 15 min at 37 °C and measured free thiols using Ellman's reagent (5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) as described [11].

Reactivity of MB with protein-bound thiols

To assess the ability of MB to bind thiols in proteins, we utilized Trx1 as a model protein. Briefly, Trx1, in its fully reduced state [12], was incubated with increasing concentrations of MB for 1 h at 37 °C. Following MB incubation, samples were desalted using a spin column (Pierce) and the free thiols were labeled using mPEG₂-biotin (Pierce), a biotinylated N-ethylmaleimide compound. The samples were then separated via SDS-PAGE, and biotinylated protein was visualized by Western blotting with fluorescently labeled streptavidin. Reactivity of MB to primary amines was also assessed using sulfo-NHS-biotin (Pierce) and application of streptavidin blotting.

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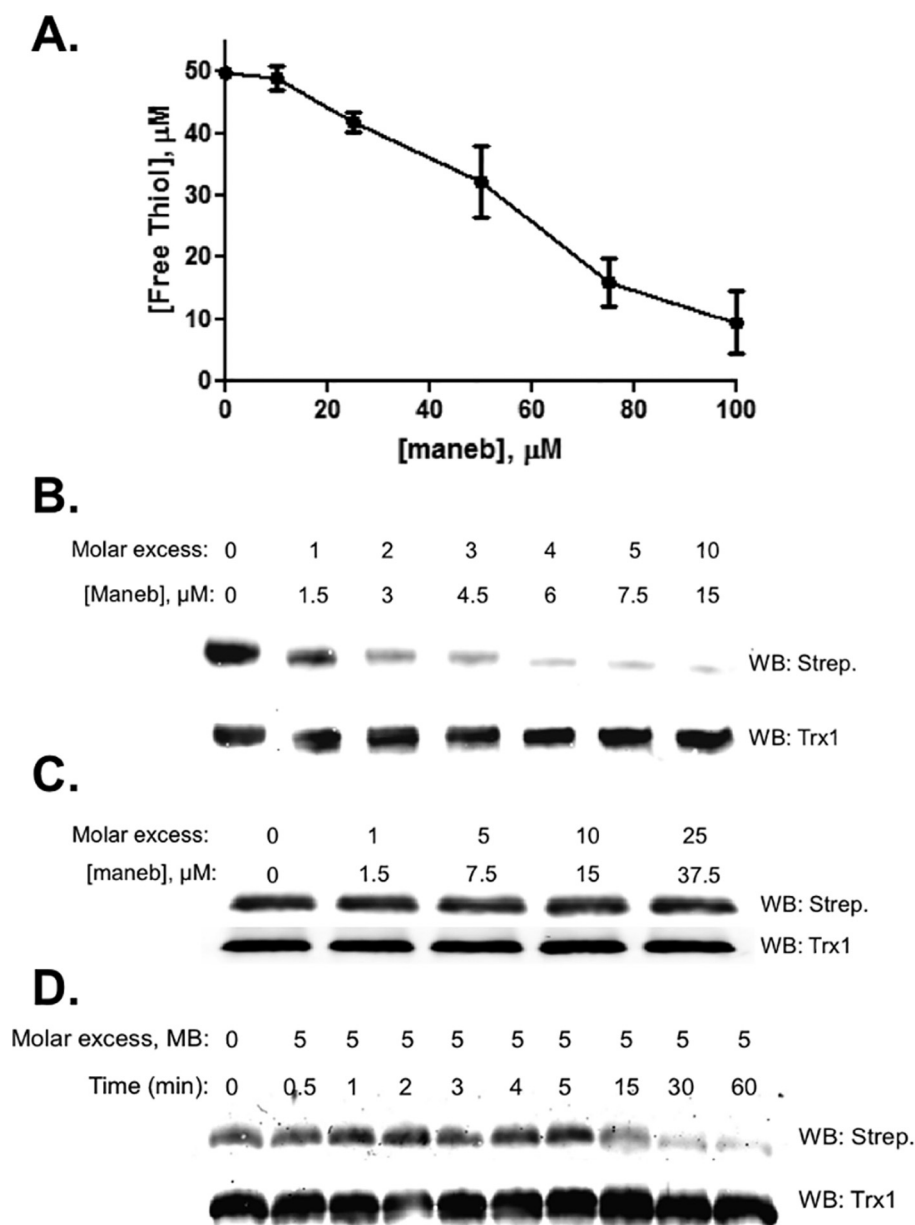


Fig. 1. Maneb (MB) is a thiol-reactive compound. (A) Addition of MB to N-acetylcysteine causes loss of thiol content as measured by Ellman's reagent. Maneb at 2:1 M ratio resulted in nearly complete loss of thiol. (B) Using thioredoxin-1 (Trx1) as a model protein, maneb stoichiometrically causes loss of protein thiols. Protein thiols labeled with mPEG₂-biotin, a biotinylated N-ethylmaleimide, and visualized in Western blot analysis with fluorescently labeled streptavidin. (C) MB does not modify amines as detected by reaction of free amines with sulfo-NHS-biotin and subsequent Western blot analysis with fluorescently labeled streptavidin. (D) A time course demonstrates that maneb causes loss of most free thiols within 15–30 min.

Trx1 activity assay

To understand the functional consequences of thiol modification by MB, Trx1 activity was assessed using the spectrophotometric insulin reduction assay described by Arner and Holmgren [13].

LC-MS analysis of intact protein

MB modification of Trx1 was assessed using intact protein LC-MS. Unmodified and MB-adducted Trx1 protein was analyzed using an Accela-LTQ Orbitrap-Velos mass spectrometer. A 10 μl injection was applied to a C18 column (5 μm , 100 \times 2.1 mm) and samples were eluted using a formic acid/acetonitrile gradient. Electrospray ionization was used in the positive mode. Raw spectra were deconvoluted utilizing a procedure in the Xcaliber program (Thermo).

Results

In this report, we used N-acetylcysteine (NAC) and the thiol-containing redox protein, human thioredoxin-1 (Trx1), to test whether MB is thiol-reactive and covalently modifies protein thiols. Results (Fig. 1A) show that free thiol concentration decreased as a function of MB concentration with a complete loss when MB:NAC reached 2:1. A kinetic assay showed that the apparent 2nd order rate constant was $5.03 \text{ M}^{-1} \text{ s}^{-1}$ (data not shown). We next examined the reactivity of MB with protein thiol using recombinant Trx1 as a model. This protein contains thiol residues that are solvent accessible, have a spectrum of reactivity and are essential for its biological function [12]. Fig. 1B shows a stoichiometric loss of protein thiols due to MB treatment. Trx1 contains 5 Cys residues and the biotin signal was

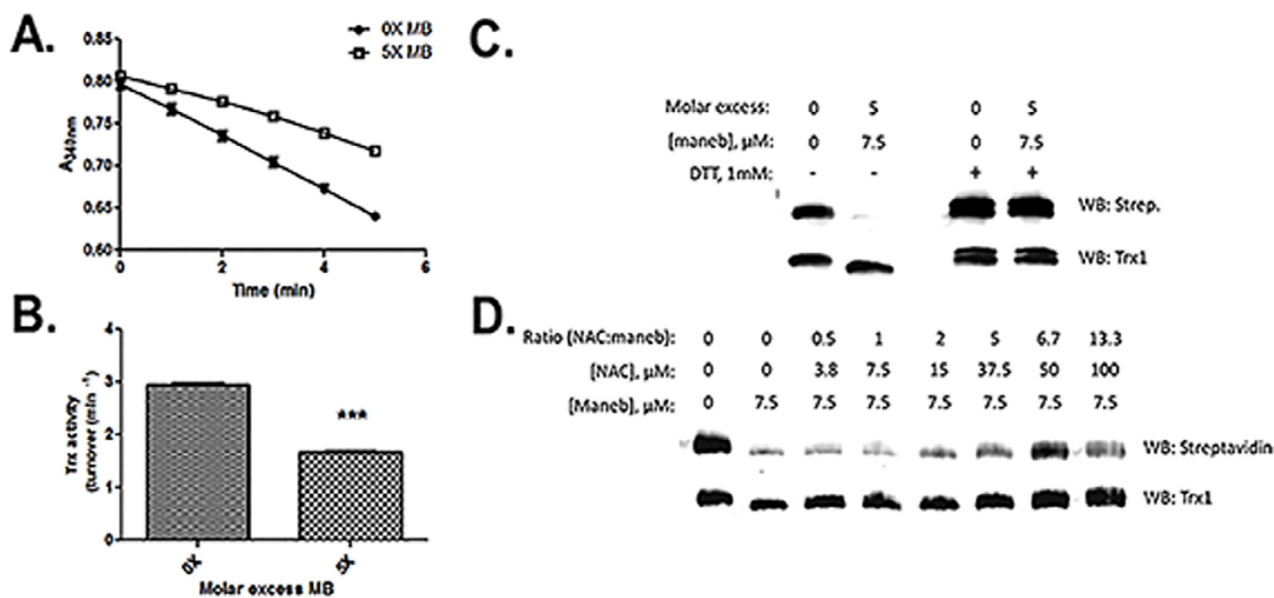


Fig. 2. Maneb inhibits thioredoxin-1 (Trx1) activity. Trx1 was treated with 5 M equivalents of mane b and activity was measured in terms of insulin reduction in the presence of NADPH and thioredoxin reductase-1. Decreased activity was evident in the rate of NADPH oxidation (A) and the calculated activity (B). Assay with DTT as the reductant instead of the NADPH/thioredoxin reductase did not show inhibition of activity (not shown). Loss of protein thiols is associated with formation of a Trx1 dimer and reversed by addition of a reductant. Both the reductant DTT (C) and N-acetyl cysteine (D) can reverse mane b-mediated loss of protein thiols.

lost when incubated with MB:Trx1 at a molar ratio of 5:1, indicating complete modification of thiols (either adduction or oxidation, see below). Reactivity of MB to primary amines was also investigated (Fig. 1C) using a procedure that is similar to visualizing protein thiols. Following MB incubation, the free amines were labeled and visualized using sulfo-NHS-biotin as demonstrated in Fig. 1B. These results show that MB does not react with amines under the conditions of the assay, preferentially modifying only protein thiols. The kinetics of the reaction was investigated with 5:1 concentration ratio using the mPEG₂-biotin method (Fig. 1D). In agreement with the NAC reactivity data, results from this experiment show that the reaction between MB and Trx1 is relatively slow, approaching complete modification of all Trx1 thiols at 60 min.

The activity of Trx1 was assessed to determine functional consequences of MB adduction. Trx1 was incubated for 1 h with MB (5:1, as above) and then desalted to remove any unreacted MB prior to the activity assay. Data show that MB modification of Trx1 slowed the Trx1-dependent oxidation of NADPH in the presence of Trx reductase by 43% (Fig. 2A and B). In contrast to these results, Trx1-catalyzed insulin reduction by DTT showed no effect on activity due to MB modification (data not shown). The incomplete inhibition of activity despite evidence for nearly complete modification of thiols indicated that MB-dependent thiol modification is likely to be reversible.

To examine reversibility, MB-modified Trx1 was incubated with 1 mM DTT, and thiol content was examined with mPEG₂-biotinylation and Western blotting as above. Results showed that the band corresponding to the unmodified thiol form of Trx1 was restored (Fig. 2C). Titration with NAC (increasing the ratio of NAC:MB from 0 to 13.3) showed that greater than a 5-fold excess of NAC was required to restore all of the thiols (Fig. 2D). The results, therefore, show that modification of Trx1 by MB is completely reversible by treatment with thiols.

X-Ray crystallography showed that oxidized Trx1 is crystallized as a dimer formed by a disulfide between C73 residues [14]. We investigated the possibility that MB treatment caused formation of a Trx1 dimer by treating Trx1 with increasing concentrations of MB, separation via SDS-PAGE under non-reducing conditions and visualization with Coomassie blue (Fig. 3A). The data demonstrate that MB results

in appearance of a band at 25 kD, corresponding to twice the molecular weight of Trx1, with as little as 1 M equivalent of MB. This result indicates that only one Cys residue is involved in the dimerization.

Due to the observation of MB-mediated protein cross-linking, we conducted LC-MS studies of intact, MB-treated Trx1 using ESI in the positive ionization mode and detection with an LTQ-Orbitrap-Velos (Thermo). These experiments resulted in the detection of Trx1 and a single modified product (Fig. 3B and C). In the MB-modified sample, we observed a large decrease in intensity of the unmodified Trx1 peak (m/z 11,602) and a new peak (m/z 11,810) caused by the binding of ethylene bis-dithiocarbamate (EBDTC) to Trx1, resulting in a mass shift of 210 mass units from the unmodified peak. This result suggests that MB does not cause a simple oxidation of thiols to disulfides but rather participates in more complex reaction processes.

Discussion

In this study we demonstrated the thiol reactivity of the dithiocarbamate fungicide MB. The data show that MB is a thiol-reactive substance that causes dimerization of Trx1 *in vitro* but does not discriminate between oxidation and more complex cross-linking that could occur through the two dithiocarbamate moieties in MB. Modification resulted in a partial inhibition of Trx1 activity, indicating that MB is either modifying active site thiol residues or peripheral residues, such as C73, that result in decreased activity. It should be emphasized again that Trx1 was employed as a model protein for these studies. Trx1 possesses thiol residues that are solvent accessible, have a spectrum of reactivity and are essential for its biological function [12].

Our data also demonstrate that the reaction between MB and thiols is relatively slow, with a rate constant ($5.03 \text{ M}^{-1} \text{ s}^{-1}$) similar to that for reaction of H_2O_2 and Trx1 ($1.05 \text{ M}^{-1} \text{ s}^{-1}$) and thiol-disulfide exchange ($20 \text{ M}^{-1} \text{ s}^{-1}$) [15,16]. Previous data show that NAC pretreatment can protect against MB-induced injury in Chinese hamster V79 cells, indicating that increased cellular thiols can protect or even prevent toxicity associated with an acute MB exposure [17]. With this in mind, the present data indicate that relatively slow, reversible binding of MB with protein thiols could trap MB in cells. Because protein thiols involved in redox signaling are more reactive than other

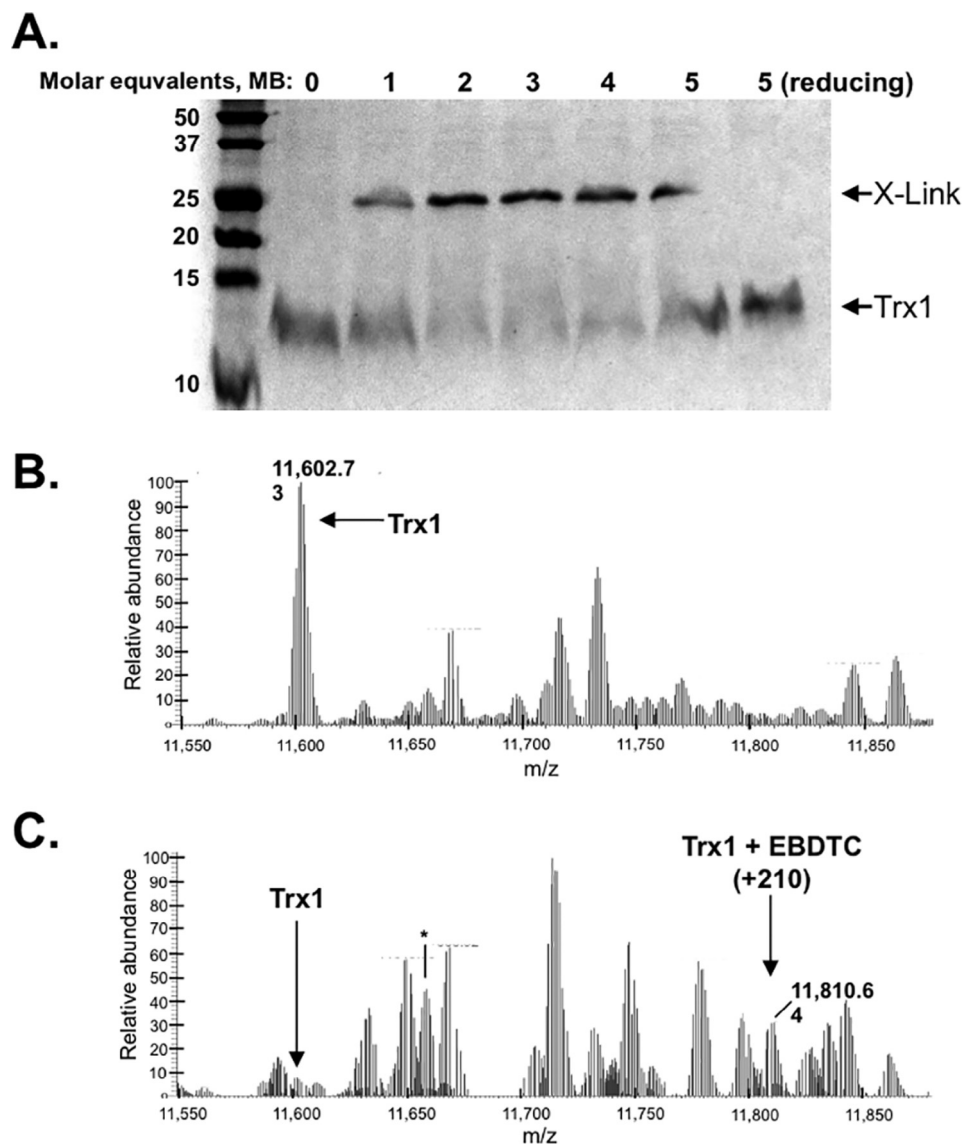


Fig. 3. MB treatment causes Trx1 to form a dimer that is reversed by DTT (A). Mass spectral analysis of intact Trx1 (B) and MB-modified Trx1 shows (C) a single MB modification (addition of 210 mass units corresponding to the addition of the ethylene bis-dithiocarbamate (EBDTC)). The asterisk (*) denotes an m/z corresponding to a potential Mn-Trx1 adduct (+54.9).

cellular thiols, such retention could allow transfer to more reactive thiols and cause prolonged disruption of redox circuits that function in redox signaling and control [18,19].

Consequently, the data suggest that MB and other similar dithiocarbamates, including mancozeb, disulfiram and zineb [20–22], may cause toxicity by disruption of redox circuits that function in cellular homeostasis. Perhaps more importantly, such interaction with critical redox signaling systems could potentiate neurotoxicity by interfering with essential cell stress response mechanisms. Although speculative, such a mechanism could explain the combined toxicity of MB and PQ in PD.

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