The Effect of *in Vitro* and *in Vivo* Ethylenbis Dithiocarbamate Fungicides on NMDA Receptors in Rat Brain Membranes

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Abstract

To determine whether the ethylenbis dithiocarbamate fungicides, zineb, manzeb and maneb affect the N-methyl-D-aspartate (NMDA) receptor in rat brain membranes, we performed a binding assay using [³H]MK-801, a noncompetitive NMDA receptor antagonist. Displacement studies were conducted using well washed membranes to exclude the effect of endogenous acidic amino acids on the binding of [³H]MK-801. In both the presence or absence of added glutamate and glycine in the assay buffer, the dose-response curve indicated that zineb enhanced the binding in a concentration range of 100–500 μ M. However, the displacement curves indicated that manzeb and maneb inhibited the binding in a concentration range of 10–500 μ M. The addition of 50 μ M glutamate and glycine to the assay medium increased binding by 5–20% above the control in a concentration range of 0.1–100 μ M.

No rats injected with zineb, manzeb, maneb (100 mg/kg, ip) showed any characteristic toxic signs or any significant weight changes within 24 hrs. Estimation of [³H]MK-801 binding to unwashed membranes from intoxicated rat brains revealed no marked change in Bmax or Kd values for 24 hrs following fungicide administration.

Key words: zineb, maneb, manzeb, NMDA receptor, neurotoxicity

Introduction

Noncholinesterase-inhibiting carbamates, dithiocarbamates, can be divided into 4 subclasses; methyl dithiocarbamates, dimethyl dithiocarbamates (DMDC), diethyl dithiocarbamates and ethylenbis dithiocarbamates (EBDC). EBDC compounds including zineb, manzeb and maneb are widely used as agricultural fungicides¹⁾. Following a large dose of zineb or maneb, rats and mice showed ataxia and hyperactivity followed by inactivity, loss of muscular tone, and alopecia. Most deaths occurred within 1–2 days²⁾. Male rats exposed to manzeb at a concentration of 100 mg/m³ for 10 days over a 2-week period showed a significant depression of mean body weight gain³⁾. Although EBDCs have been used because of their low acute toxicity in mammals, the EBDCs and their metabolites, carbon disulfide and ethylene thiourea (ETU), have been reported as teratogens in the central nervous system (CNS)³⁾. In humans, there has been only one report of behavioral and CNS changes due to acute exposure to Manzidan (a combined dithiocarbamate of maneb and zineb)⁴⁾, and all symp-

toms disappeared after a few days in that case. Maneb and, more generally, dithiocarbamates, have been reported to induce a permanent extrapyramidal syndrome resembling Parkinsonism after prolonged exposure^{5–7)}.

It was recently reported that the EBDC compounds, manzeb and maneb are suspected environmental endocrine disruptors⁸⁾. There is increasing evidence that some environmental chemicals can interrupt neurodevelopment processes during critical periods of development, which can effect sensory, motor, and cognitive function⁹⁾. Behavioral changes can be caused by exposure to maneb during the postnatal period at levels approximating food residue levels, but well below acute toxic doses¹⁰⁾. Despite increased knowledge of the neurotoxicity of EBDC compounds, relatively few studies have been carried out on the effects of carbamates on neuroreceptors in connection with behavioral changes.

There is now ample evidence in the literature to suggest that the NMDA (N-methyl-D-aspartate)-receptor, a subtype of the glutamate receptor, is directly involved in learning and memory functions through synaptic plasticity and long term potentiation¹¹⁾. Furthermore, numerous studies in experimental animals indicate the involvement of the NMDA-receptor in several neurological diseases¹²⁾. The aim of the present study was to clarify the *in vitro* and *in vivo* effects of the EBDC fungicides, zineb, maneb and manzeb on NMDA receptors in the rat brain. We carried out binding assays using [³H]MK-801 as a radioligand. Noncompetitive

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NMDA antagonists, such as dizocilpine (MK-801) and phencyclidine, impair performance in a variety of learning and memory tasks¹³). Therefore, the present binding studies provided information about the functional state of the NMDA-receptor complex.

Materials and Methods

Chemicals

[³H]MK-801 (specific activity: 22.5 Ci/mmol, concentration: 1.0 mCi/ml) were obtained from NENTM Life Science Products, Inc. (Boston, MA, USA). (+)-MK-801 hydrogen maleate was obtained from RBI (Natick, MA, USA). Manzeb (Mancozeb, Manganese Ethylenebis (dithiocarbamate) (polymeric) complex with Zinc Salt, purity 91.0%¹), Maneb (Manganese Ethylenebis (dithiocarbamate) (polymeric), purity 90.2%) and Zineb (Zinc Ethylenebis (dithiocarbamate) (polymeric), purity 82.1%) were obtained from Wako Pure Chemical Industries, Ltd. Osaka, Japan. All other chemicals were from commercial sources.

Animals and treatment

Female Sprague-Dawley rats (9–10 weeks) weighing 200–220 g were housed in groups of four animals per cage. They were maintained under conditions of 21±2°C and a 12–12 hr light-dark cycle with free access to food and water for a week before treatment or sacrifice. For the *in vitro* study, control rats, which received no particular treatment were used. For the *in vivo* study, four groups were established each consisting of 4 rats. One group served as a control. Three of the groups were administered Zineb, Manzeb and Maneb, respectively. Fungicides were suspended in 1% arabic gum and given intraperitoneally at 100 mg/2 ml/kg body weight. Control animals received a vehicle (1% arabic gum in deionized water) at 2.0 ml/kg body weight. After 24 hours, the rats were euthanized by decapitation. The brain was rapidly removed and the cerebral cortex dissected. The animal protocols were reviewed and approved by the Fukushima Medical University Animal Care and Use Committee.

Tissue preparation

Rat brain membranes were prepared from the cerebral cortices of female Sprague-Dawley rats according to the procedure described by Ransom and Stec¹⁴). Briefly, the rat cerebral cortices were dissected and homogenized in a 10 vol (weight/vol) of ice-cold 0.32 M sucrose using a Teflon homogenizer. The homogenates were centrifuged at 1,000 g for 10 mins, and the supernatant was recentrifuged at 18,000 g for 20 mins. The pellet was resuspended and homogenized using Polytron in a 10 vol of 5 mM Tris-HCl (pH 7.7 at 4°C) followed by centrifugation at 8,000 g for 20 mins. The supernatant and the buffy coat of the pellet were transferred to centrifuge tubes and centrifuged at 50,000 g for 20 mins. The pellet was resuspended in a 10 vol of 5 mM Tris-HCl, pH 7.7, homogenized with Polytron, and centrifuged at 50,000 g for 20 mins. For “unwashed membrane” preparations, the pellet was resuspended in 5 mM Tris-HCl, pH 7.5 (assay buffer) and stored at –75°C until use. This preparation was used as an assay sample without further washing. For “washed membrane” preparations, the wash step was repeated twice more, and the final pellet was stored at –75°C for at least 18 hours. On the day of assay, the “washed membrane pellets” were resuspended in a 20 vol of 5 mM Tris-HCl, pH 7.7, homogenized with the Polytron, and centrifuged at 50,000 g for 20 mins. This washing step was repeated

four more times and the final pellet was resuspended in an assay buffer (5 mM Tris-HCl, pH 7.5, at 25°C). Thus, the “unwashed membranes” underwent 5 centrifugation/resuspension cycles while the “washed membranes” underwent 11 cycles.

Binding assays

Solutions of the fungicides for binding studies were made in dimethyl sulfoxide (DMSO) and were diluted in an assay buffer (pH 7.5). The DMSO concentrations of the final reaction mixtures were less than 5% and the pH range was between 7.4 and 7.5. For the saturation analysis, the washed membranes (100–200 µg protein/well) were incubated with increasing concentrations of [³H]MK-801 (0.2–20 nM) in the presence of 50 µM glutamate and glycine in 96 well plastic microtiter plates. The specific binding was defined as the difference between the total binding and the non-specific binding in the presence of 50 µM unlabeled MK-801. For displacement studies, the washed membranes (100–200 µg protein/well) were incubated with 5.0 nM radiolabeled MK-801 and 0.1–500 µM of a test fungicide in the presence or absence of 50 µM glutamate and glycine in microtiter plates. When the saturation and displacement studies were carried out using unwashed membranes, the binding assays were performed without added glutamate or glycine in the reaction mixture. In all studies, the reaction mixtures were incubated in a total volume of 250 µl binding buffer (5 mM Tris-HCl, pH 7.5) for 60 mins at room temperature (21–23°C). The reaction was terminated by rapid filtration over a glass-fiber filter (Whatman GF/B filters) using an INOTECH (Switzerland) cell harvester, followed by two washes with an ice-cold buffer. Filters were placed in counting vials, 2 ml scintillation fluid was added and radioactivity was counted in an Aloka model 3,500 liquid scintillation analyzer.

Data analysis

Saturation, binding and displacement data were analyzed with a non-linear least squares curve fitting, using PriSm software (ver.2.0) for the Macintosh-CP (GraphPad, San Diego, CA, USA).

Protein determination

Protein measurements in membrane preparations were done using the method of Bradford¹⁵) with a Bio-Rad Protein Assay Kit (Nippon Bio-Rad Laboratories, Tokyo, Japan).

Statistics

The control and treatment group data were compared by one-way analysis of variance (ANOVA) using StatView for Macintosh, ver.5.0. (SAS Institute Inc. Cary, NC, USA).

Results

Chemicals

The chemical structure and molecular weight of manzeb (mancozeb), maneb and zineb are shown in Fig. 1. Manzeb (mancozeb) is a manganese ethylenebis(dithiocarbamate) (polymeric) complex with zinc salt. The ISO definition is a “complex of zinc and maneb containing 20% manganese and 2.55% zinc, the salt present being stated (for instance, manzeb chloride)”²). In the present study, we defined the molecular weight of manzeb as 271.8 using x=1 and y=0.1. Thus, manzeb contained 20.7% manganese and 2.4% zinc. The molecular weight of maneb was 265.3 and it contained 20.7% manganese. The molecular weight of zineb

Fig. 1 Chemical structures of manzeb, maneb and zineb.

was 275.7 and it contained 23.6% zinc.

In vitro experiments

Saturation analysis of specific [^3H]MK-801 binding to well washed rat cerebral cortex membranes revealed a single saturable class of binding sites with a dissociation constant (K_d) of 11.25 ± 3.14 nM and a maximal binding capacity (B_{max}) of 2.21 ± 0.60 pmol/mg protein (mean $\pm$ SD, three experiments). A representative saturation curve (the B_{max} and K_d values were 2.31 pmol/mg protein and 12.30 nM, respectively) is shown in Fig. 2. Fig. 3 shows that zineb increased binding in a concentration range of 100–500 μM , both with and without adding glutamate and glycine to the medium. Zineb enhanced [^3H]MK-801 binding at concentrations up to 500 μM . Because the binding reaction did not reach a plateau at the highest concentration, EC_{50} values were not obtained under these assay conditions. About 10–20% of specific [^3H]MK-801 binding was enhanced by the addition of 50 μM

Fig. 2 Representative saturation isotherm and Scatchard plot (inset) showing [^3H]MK-801 binding to the washed membranes. Each point is the mean of duplicate assays from a single representative experiment. The estimated B_{max} and K_d values were 2.31 pmol/mg protein and 12.30 nM, respectively.

Fig. 3 Effect of zineb on [^3H]MK-801 binding to rat brain membranes. Binding assays were performed as described in Methods using washed membranes from the cerebral cortex. Dose-response curves were generated in the absence of amino acids (squares) or presence (triangles) of 50 μM glutamate and glycine. Data points are means of three determinations performed in duplicate.

glutamate and glycine in a concentration range of 0.1–400 μM . Fig. 4 demonstrates that manzeb inhibited binding in a concentration range of 10–500 μM . Analysis of the binding data indicated that the inhibition constant (K_i) values in the absence of amino acids, and in the presence of glutamate and glycine, were 321 μM and 322 μM , respectively. Maneb also inhibited binding in the same concentration range (Fig. 5). The K_i values in the absence of amino acids and in the presence of glutamate and glycine were 806 μM and 427 μM , respectively. Slight stimulation of 5–10% above the control was observed, with the addition of the amino acids, in both fungicides.

In order to investigate the effects of the fungicides on [^3H]MK-801 binding to unwashed membranes, displacement experiments were carried out in the absence of added glutamate and glycine in a concentration range between 0.1 and 500 μM . Fig. 6 shows that zineb stimulated binding in a concentration range of 50–500 μM . In contrast, manzeb and maneb inhibited binding over the same concentration range.

Fig. 4 Effects of manzeb on [^3H]MK-801 binding to rat brain membranes. Binding assays were performed using washed membranes from the cerebral cortex. Displacement curves were generated in the absence of amino acids (squares) or presence (triangles) of 50 μM glutamate and glycine. Data points are means of three determinations performed in duplicate. The inhibition constant (K_i) values in the absence and presence of glutamate and glycine were 321 μM and 322 μM , respectively.

Fig. 5 Effects of manebe on [^3H]MK-801 binding to rat brain membranes. Binding assays were performed using washed membranes from the cerebral cortex. Displacement curves were generated in the absence of amino acids (squares) or presence (triangles) of 50 μM glutamate and glycine. Data points are means of three determinations performed in duplicate. The K_i values in the absence of amino acids and presence of glutamate and glycine were 806 μM and 427 μM , respectively.

Fig. 6 Effects of zineb, manzeb and manebe on [^3H]MK-801 binding to rat brain membranes. Binding assays were performed using unwashed membranes as described in Methods. Dose-response curves were generated with no addition of glutamate or glycine to the assay buffer. Each point is mean of triplicate assays from a single experiment. The estimated K_i values were 335 μM for manzeb and 507 μM for manebe. The control value was 1.36 pmol/mg protein (mean of 9 assays).

In vivo experiment

No rats injected intraperitoneally with 100 mg/kg body weight of zineb, manzeb or manebe showed any characteristic toxic signs or significant weight changes within 24 hrs (data not shown).

Table 1 Scatchard analysis of [^3H]MK-801 binding to cerebral cortex membranes from rats treated with fungicides

Treatment	Dose (mg/kg body wt.)	Bmax (pmoles/mg protein)	Kd (nM)
Control	vehicle	1.39 $\pm$ 0.59	3.41 $\pm$ 1.83
Zineb	100	1.05 $\pm$ 0.62	2.13 $\pm$ 1.53
Manzeb	100	1.44 $\pm$ 1.04	3.44 $\pm$ 2.45
Maneb	100	1.40 $\pm$ 0.76	2.86 $\pm$ 0.89

Rats were exposed to the indicated treatments and decapitated 24 hrs after the ip injection. There were four rats in each group. Results shown are the mean $\pm$ SD of four experiments performed in duplicate.

Scatchard analysis of MK-801 binding to unwashed membranes demonstrated no significant decrease or increase in the Bmax and Kd values of the cerebral cortex in the fungicide-treated animals (Table 1). Although the mean Bmax and Kd values of zineb injected decreased 25% and 38% compared with the control (vehicle treated group), the differences were not significant.

Discussion

The difference in [^3H]MK-801 binding among brain regions suggested a difference in NMDA receptor distribution in the brain^{16,17}. In the present study, cerebral cortex membranes were used, since the cerebral cortex is the largest brain region with a high density of NMDA receptors.

The saturation analysis of [^3H]MK-801 binding shown in Fig. 2 is consistent with the observation of Wong et al.¹⁶, although [^3H]MK-801 bound with a slightly lower affinity in the present study compared to their results. In both studies, the binding experiments were carried out using well washed membranes in the presence of L-glutamate and glycine in an assay medium. A reduction in the levels of endogenous glutamate and glycine by washing was needed to observe the effects of these amino acids¹⁸. Exogenous L-glutamate and glycine stimulate the binding of [^3H]MK-801 to extensively washed brain membranes^{14,19}. The addition of glutamate (100 μM) and glycine (30 μM) increased [^3H]MK-801 binding to 200% above that of the control²⁰. The present study showed that addition of amino acids was much less effective (5–20% above) (Fig. 3–5), but since the ligand concentration used in the present assay (5 nM) was higher than the previous study (0.5–1 nM), this may account for the relatively low effect of the amino acids observed. The enhancement of binding by exogenous L-glutamate and glycine was due solely to an increase in the affinity of [^3H]MK-801¹⁴. Thus, the effect of exogenous amino acids on binding was more marked at low ligand levels rather than at high levels.

In the present *in vitro* study, the binding of the ligand [^3H]MK-801 to brain membranes increased with zineb, and decreased with manzeb and manebe. These fungicides may interrupt the function of the NMDA receptor in the brain. In previous studies, Zn^{2+} invariably elicited an inhibitory action on [^3H]MK-801 binding independent of the membrane preparations used^{16,20–22}. In contrast, zineb enhanced the binding in both the washed and unwashed membrane preparations (Fig. 3, 6). Thus, it is unclear whether zinc, which is the metal substituent in the zineb molecule, is related to the modulation process. The manganese component of manzeb (a coordinated product of zinc ions and manebe, containing 20% manganese and 2.5% zinc) seemed to have some inhibitory effects, because manebe (manganese ethylenebis dithiocarbamate) also inhibited the binding (Fig. 5, 6). Mn^{2+} has an inhibitory effect on [^3H]MK-801 binding in brain membranes that have not been extensively washed, but a stimulatory effect in membranes that have been extensively washed¹⁶. Since the present results were obtained from binding experiments using not only washed, but also unwashed membranes, further investigation is required to elucidate the action of the manganese component in the manzeb and manebe molecules.

MK-801 was reported to be a potent and selective non-competitive blocker of NMDA channels. Access of [^3H]MK-801 to its binding site inside the channel is controlled by drugs that act on the NMDA receptor and modify the opening of the channel.

Among several divalent cations, Pb^{2+} was found to be the most potent in inhibiting the binding of [3H]MK-801. The blocking action of Pb^{2+} on the NMDA receptor ion channel is an important clue for the further investigation of clinical effects, such as deficits in cognitive development and learning disorders, of this cation^{23,24}). Therefore, the inhibitory effects of maneb and manzeb on [3H]MK-801 binding suggest that these compounds are neurotoxic, and may be related to behavioral changes.

In order to investigate the change in [3H]MK-801 binding to NMDA receptors in the intoxicated rat brain, we performed a preliminary binding assay without adding glycine and glutamate using unwashed membranes prepared from control rats. Under these conditions [3H]MK-801 binding was maximally stimulated by endogenous L-glutamate and L-aspartate i.e., the activated state of the NMDA receptor¹⁹). Metabolite(s), which may affect binding also remain in the unwashed membranes. As shown in Fig. 5, zineb induced increased binding, while manzeb and maneb induced decreased binding. These stimulatory or inhibitory effects on binding to unwashed membranes were comparable with results

obtained from washed membranes (Fig. 3–5). The [3H]MK-801 binding properties (K_d and B_{max}) to unwashed membranes observed under the present conditions were also consistent with the results reported by Wong et al.¹⁶). These results suggest that unwashed membranes are useful for detecting the *in vivo* effect of fungicides on NMDA receptors.

In our *in vivo* experiments, the EBDC fungicides at an ip dose of 100 mg/kg failed to produce significant effects on NMDA receptor properties (Table 1). Olney et al.²⁵) reported that there was an apparent neurotoxic effect due to MK-801 and related agents (phencyclidine, tiletamine, and ketamine). These agents induce acute pathomorphological changes in specific populations of brain neurons when administered subcutaneously to adult rats. The EBDC fungicides have very low toxicity with oral LD50 values in the rat of >5,000 mg/kg for zineb, 8,000 mg/kg for manzeb and 6,750 mg/kg for maneb²). Further large-dose experiments are required to elucidate the *in vivo* effects of these fungicides on NMDA receptors.

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