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The ethylene bis-dithiocarbamate fungicide Mancozeb activates voltage-gated KCNQ2 potassium channel

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HIGHLIGHTS

- Mancozeb is a neuronal KCNQ2 potassium channel activator.
- Metal ions are critical for the potentiation effect.
- The metal-containing EBDC fungicides potentiate KCNQ2 channel as a specific complex.

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ABSTRACT

Mancozeb (manganese/zinc ethylene bis-dithiocarbamate) is an organometallic fungicide that has been associated with human neurotoxicity and neurodegeneration. In a high-throughput screen for modulators of KCNQ2 channel, a fundamental player modulating neuronal excitability, Mancozeb, was found to significantly potentiate KCNQ2 activity. Mancozeb was validated electrophysiologically as a KCNQ2 activator with an EC_{50} value of 0.92 ± 0.23 μM. Further examination showed that manganese but not zinc ethylene bis-dithiocarbamate is the active component for the positive modulation effects. In addition, the compounds are effective when the metal ions are substituted by iron but lack potentiation activity when the metal ions are substituted by sodium, signifying the importance of the metal ion. However, the iron (Fe^{3+}) alone, organic ligands alone or the mixture of iron with the organic ligand did not show any potentiation effect, suggesting as the active ingredient is a specific complex rather than two separate additive or synergistic components. Our study suggests that potentiation on KCNQ2 potassium channels might be the possible mechanism of Mancozeb toxicity in the nervous system.

1. Introduction

As a metal-containing ethylene bis-dithiocarbamate (EBDC) fungicide, Mancozeb has been wildly used in agriculture to control a variety of fungal infections in vegetables and ornamental plants. Mancozeb is a complex mixture of manganese EBDC (Mn-EBDC, also named Maneb) and zinc EBDC (Zn-EBDC, also named Zeneb) (Cambridge soft corporation, chemical abstracts, index guide [EB/OL]: http://chemfinder.cambridgesoft.com). Both Mn-EBDC and Zn-EBDC are also used as fungicides. These fungicides have been thought to show little acute toxicity in humans and thus are used widely throughout the world (Corsini et al., 2005). Mancozeb is one of the most commonly applied fungicide in the United States. The total amount of Mancozeb and Mn-EBDC applied in the United States in 2009 was around at 3.4 million pounds (USGS.gov, 2009). The major toxicological concern from exposure to Mancozeb is the hazard to the human thyroid and carcinogenic effects in test animals (Axelstad et al., 2011; Belpoggi et al., 2002; Goldner et al., 2010; Nicolau, 1982; Nordby et al., 2005; Steenland, 2003; Steenland et al., 1997; Trivedi et al., 1993).

However, a number of recent studies suggest Mancozeb affects the human nervous system. Mancozeb has been associated with human neurotoxicity and neurodegeneration, such as manganism and parkinsonism (Debbarh et al., 2002; Domico et al., 2007,

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2006; Kirrane et al., 2005; McCormack et al., 2002; Negga et al., 2012; Vaccari et al., 1999). The pathology of Parkinson's syndrome includes damage of dopamine (DA)-ergic neurons and γ-aminobutyric acid (GABA)-ergic neurons (Barone, 2010; Kim et al., 2002; Langston, 2006; Mhyre et al., 2012; Surmeier and Sulzer, 2013; Willis et al., 2012). It has been reported that DAergic neurons are vulnerable to Maneb exposure (Cicchetti et al., 2005; Costello et al., 2009; Domico et al., 2006; Meco et al., 1994). More recently, a study in the model organism *C. elegans* showed that exposure to Mancozeb promotes neurodegeneration in both GABAergic and DAergic neurons (Negga et al., 2012). Interestingly, another more recent study in *C. elegans* showed that Mancozeb-induced behavioral deficits precede structural neural degeneration (Harrison Brody et al., 2013). Generation of reactive oxygen species (ROS), auto-oxidation of catecholamines, alterations in antioxidant defense systems and inhibition of cholinesterase by Mancozeb have been reported, which may contribute to the neuronal toxicity effects (Ahmad et al., 2010; Barlow et al., 2005; Bhat et al., 2010; Domico et al., 2007, 2006; Fitsanakis et al., 2002; Grosicka-Maciag et al., 2011; Jarrard et al., 2004). For humans, exposure to Mancozeb causes acute and long-term consequences (Delgado and Paumgartten, 2004). The neurotoxicity symptoms include fatigue, headache, blurred vision and nausea. At high doses, exposed persons may experience convulsions, slurred speech, confusion, etc. (www.epa.gov).

KCNQ2 potassium channel (Kv7.2) is a low-threshold voltage-gated potassium (Kv) channel. As a primary player that mediates neuronal muscarinic (M) currents, opening of KCNQ2 channel or of heterogeneous KCNQ2 complexes will inhibit initiation of action potential and thus dampen neuronal excitability (Robbins, 2001). In contrast, inhibition of the channel causes hyperexcitability (Brown and Passmore, 2009; Piccinin et al., 2006; Yue and Yaari, 2004). Reduction or loss of KCNQ2 channel activity is related with a single type of familial convulsion (Charlier et al., 1998; Singh et al., 1998). In the current study, we report Mancozeb potentiates KCNQ2 channel in a dose-dependent manner. We also show that Mn-EBDC, while not Zn-EBDC, is the active component for the potentiation effect. To our knowledge, this is the first report about the effects of Mancozeb on ion channels.

2. Materials and methods

2.1. Chemicals

Mancozeb (Purity: 96.4%; MW: 266.5), Zn-EBDC (Purity: 92.7%; MW: 275.8) manganese chloride tetrahydrate ($MnCl_2$; MW 197.90), MTT reagent, tetraethoxypropane (TEP) and sodiumpyruvate were purchased from Sigma–Aldrich (St. Louis, MO, USA). Mn-EBDC (Purity: 85%; MW: 265.3) and 2Na-EBDC (Purity: 69%; MW: 256.4) were purchased from J&K scientific (Shanghai, China). Aqueous Amobam (45%) was purchased from ShuangJi Chemicals (Hebei, China). All reagents were used without further purification. Nuclear magnetic resonance (NMR) spectroscopy was performed on a Bruker AMX-400 NMR, using TMS as internal standard and pyridine-d_6 as solvent. Elemental analyses were carried out by the Chemical Analysis Laboratory at SIMM (CAS, China).

2.2. Organic synthesis

2.2.1. Preparation of organic ligand (2H-EBDC)

Carbon disulfide (0.096 mol) was added dropwise to a mixture of ethylenediamine (0.04 mol) in water (5 mL), keeping the temperature below 10 °C for 2 h. The solid product obtained was filtered and washed with ethanol. After drying under vacuum, a white power was obtained. Yield 70%, ^{1}H NMR (400 MHz, pyridine): δ 3.57 (s, 4H), 9.18 (s, 2H).

2.2.2. Preparation of metal complexes

To a stirred mixture of $FeSO_4{\cdot}7H_2O$ (0.04 mol) and H_2O (100 mL) at 30 °C under N_2, 45% aqueous Amobam ($2NH_4$-EBDC, 0.04 mol) was added. The resulting solution was stirred for 1 h. The precipitate was filtered, washed successively with H_2O and ethanol, followed by drying under vacuum to yield Fe^{2+}-EBDC·$3H_2O$ as brown powder. Yield 91%. Anal. Calcd. for $C_4H_6FeN_2S_4$-$3H_2O$: C, 15.00; N, 8.75; S, 40.05; Fe, 17.44. Found: C, 15.24; N, 8.87; S, 40.19; Fe, 17.70.

$2Fe^{3+}$-3EBDC·$6H_2O$ was obtained in the same manner described in the preparation of Fe^{2+}-EBDC·$3H_2O$ from $FeCl_3{\cdot}6H_2O$ and 45% aqueous Amobam. Yield: 89%. Anal. Calcd. for $C_{12}H_{18}Fe_2N_6S_{12}$-$6H_2O$: C, 16.94; N, 9.88; S, 45.22; Fe, 13.13. Found: C, 16.69; N, 9.88; S, 44.20; Fe, 15.10.

2.3. Cell culture and transfection

CHO (Chinese hamster ovary) cells were grown in 50/50 DMEM/F-12 (Life Technologies, Carlsbad, CA, USA) with 10% FBS (fetal bovine serum), and 2 mM L-glutamine (Life Technologies, Carlsbad, CA, USA). Cells were split at 24 h before transfection, plated in 60-mm dishes, and transfected with Lipofectamine 2000™ reagent (Life Technologies, Carlsbad, CA, USA) to express the channels, according to the manufacturer's instructions. To identify the transfected cells by fluorescence microscopy, a GFP cDNA (Amaxa, Gaithersburg, MD, USA) was cotransfected. The KCNQ2 cDNA was a gift from M. Sanguinetti (University of Utah).

2.4. FluxOR thallium assay

The rat KCNQ2 channel stable CHO cell line was routinely cultured in DMEM/F-12 medium, supplemented with 10% FBS and 500 mg/mL G418. CHO-KCNQ2 cells were seeded in 96-well plates at ~10,000 cells/well and cultured until 80–90% confluence at 37 °C in a 5% CO_2 incubator. The medium was removed the next day and 80 μL of FluxOR loading buffer was added to each well for 90 min at room temperature (RT) in darkness. Once the loading buffer was removed, 100 μL/well of assay buffer and 20 μL/well of 7× control/test compound was added to cells at RT in darkness. The tested compounds were prepared using assay buffer; controls were assay buffer representing EC_0 and ztz240 representing EC_{100}. 30 min later, cell plates were loaded on FDSS. After 10 s of recording, 20 μL/well of stimulus buffer was added. The plates were read every second for 110 s. The stimulus buffer contained 1.30 mM K_2SO_4 and 9.80 mM Tl_2SO_4. The FluxOR thallium assay protocol above is identical to the manufacturer's protocol. The △fluorescence potentiation (short as Fluo. Potenti.) = $(R_{test} - R_{control})/(R_{control} - R_{buffer}) \times 100\%$% was calculated for each well using the 35 s fluorescence ratio. In order to identify compounds with potentiation activity on KCNQ channels, a thallium flux assay was developed and used to screen a lab library of 324 compounds at 10 μM final concentration.

2.5. Electrophysiology recording in CHO cells

Standard whole-cell recording was used to record current of the transiently expressed KCNQ channels in CHO cells. Pipettes were pulled from borosilicate glass capillaries (TW150-4, World Precision Instruments, Sarasota, FL, USA). When filled with the intracellular solution, the pipettes have 3–5 MΩ resistance. During the recording, extracellular solution was constantly perfused by a BPS perfusion system (ALA Scientific Instruments, Farmingdale, NY, USA). Intracellular solution contained (in mM): 145 KCl, 1 $MgCl_2$, 5 EGTA, 10 HEPES and 5 MgATP (pH = 7.3); extracellular solution contained (in mM): 140 NaCl, 3 KCl, 2 $CaCl_2$, 1.5 $MgCl_2$, 10 HEPES and 10 glucose (pH = 7.4). Both current and voltage were recorded using an Axopatch-200B amplifier, filtered at 2 kHz, and digitized using a DigiData 1440A with pClamp 10.2 software (Axon Instruments). Series resistance compensation was also used and set to 60–80%.

2.6. Data analysis

Patch clamp data were processed using Clampfit 10.2 (Molecular Devices, Sunnyvale, CA, USA) and then analyzed in GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Voltage-dependent activation curves were fitted with the Boltzmann equation, $G = G_{min} + (G_{max} - G_{min})/(1 + \exp(V - V_{1/2})/S)$, where G_{max} is the maximum conductance, G_{min} is the minimum conductance, $V_{1/2}$ is the voltage for reaching 50% of maximum conductance, and S is the slope factor. Dose–response curves were fitted with the Hill equation, $E = E_{max}/(1 - (EC_{50}/C)P)$, where EC_{50} is the drug concentration producing half of the maximum response, and P is the Hill coefficient. Data are presented as means ± S.E.M. Significance was estimated using unpaired two-tailed Student's t tests. An effect was considered significant if $p < 0.05$.

3. Results

3.1. Mancozeb potentiates KCNQ2 channel

To identify compounds with potentiation effects on KCNQ channels, we generated a KCNQ2 stable cell line. After a series of optimizations for several key screening parameters, a standard procedure of thallium flux assay was developed and used to screen an in-house collection of over 300 approved drugs at 10 μM final concentration. In the pilot screen, Mancozeb exhibited comparable potentiation to the positive control compound, ztz240, as observed by the fluorescence signal (Fig. 1A). The structure of Mancozeb is shown in Fig. 1B.