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4R-cembranoid protects against diisopropylfluorophosphate-mediated neurodegeneration

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ABSTRACT

Many organophosphorous esters synthesized for applications in industry, agriculture, or warfare irreversibly inhibit acetylcholinesterase, and acute poisoning with these compounds causes life-threatening cholinergic overstimulation. Following classical emergency treatment with atropine, an oxime, and a benzodiazepine, surviving victims often suffer brain neurodegeneration. Currently, there is no pharmacological treatment to prevent this brain injury. Here we show that a cyclic diterpenoid, (1S,2E,4R,6R,7E,11E)-cembra-2,7,11-triene-4,6-diol (4R) ameliorates the damage caused by diisopropylfluorophosphate (DFP) in the hippocampal area CA1.

DFP has been frequently used as a surrogate for the warfare nerve agent sarin. In rats, DFP is lethal at the dose used to cause brain damage. Therefore, to observe brain damage in survivors, the death rate was reduced by pre-administration of the peripherally acting antidotes pyridostigmine and methyl atropine or its analog ipratropium. Pyridostigmine bromide, methyl atropine nitrate, and ipratropium bromide were dissolved in saline and injected intramuscularly at 0.1 mg/kg, 20 mg/kg, and 23 mg/kg, respectively. DFP (9 mg/kg) dissolved in cold water was injected intraperitoneally. 4R (6 mg/kg) dissolved in DMSO was injected subcutaneously, either 1 h before or 5 or 24 h after DFP. Neurodegeneration was assessed with Fluoro-Jade B and amino cupric silver staining; neuroinflammation was measured by the expression of nestin, a marker of activated astrocytes.

Forty-eight hours after DFP administration, 4R decreased the number of dead neurons by half when injected before or after DFP. 4R also significantly decreased the number of activated astrocytes. These data suggest that 4R is a promising new drug that could change the therapeutic paradigm for acute poisoning with organophosphorous compounds by the implementation of a second-stage intervention after the classical countermeasure treatment.

1. Introduction

Organophosphorous compounds (OPs) are a diverse family of natural and man-made compounds used in industry, in agriculture as insecticides, and most notoriously, in warfare and terrorism as chemical warfare nerve agents (CWNA). OPs that inhibit acetylcholinesterase (AChE) (Pope et al., 2005) or the neuropathy target esterase (Damodaran et al., 2011) pose a threat to human life and health. Chronic exposure to low quantities of these compounds produces pathologies that appear to be less dependent on AChE inhibition than acute poisoning (Ray and Richards, 2001; Terry et al., 2011; Terry, 2012). In the case of acute OP poisoning, most, but not all, neurological damage caused by these compounds can be accounted for by inhibition of AChE, accumulation of acetylcholine, and the ensuing cholinergic crisis. For the last 50 years, medical countermeasures included administration of atropine to antagonize muscarinic effects, pralidoxime to reactivate AChE, and benzodiazepine to reduce seizures. These classical antidotes are effective in decreasing mortality but are ineffective in protecting against the delayed neurotoxicity that often follows. For example,

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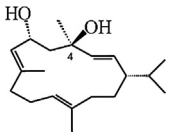

Fig. 1. Structural formula of 4R-cembranoid. The chiral carbon 4, relevant to neuroprotection, is indicated.

the victims of the Tokyo 1995 sarin incident still suffer the consequences of OP poisoning. Many survivors showed severe neurological complications 7 years after the incident (Miyaki et al., 2005). To attenuate this delayed OP-mediated neurotoxicity, one should, therefore, target later steps along the pathway leading to brain damage, including inflammation and apoptosis (Chen, 2012).

The need for a neuroprotective compound capable of ameliorating neurodegenerative processes is evident. Our group discovered a diterpenoid called 4R-cembranoid (4R) that fits in this category (Fig. 1) (Ferchmin et al., 2009). Several natural and semisynthetic analogs of 4R are also promising neuroprotective compounds (Eterovic et al., 2013).

The objective of this study was to determine whether 4R could decrease the neurodegeneration caused by diisopropylfluorophosphate (DFP) under conditions that simulate terrorist attacks or industrial accidents. DFP is an extremely toxic OP that has been used as a surrogate CWNA (Crawford et al., 2004; Deshpande et al., 2010). *In vitro* studies revealed that 4R is an antagonist of the α7 nicotinic acetylcholine receptor. Interestingly, by inhibition of this receptor, 4R triggers a nicotinic neuroprotective cell-signaling cascade (Ferchmin et al., 2005, 2013). In *vitro* studies have shown that 4R rescues the electrophysiological activity of acute hippocampal slices measured as population spikes when applied 30 min after exposure to the OP paraoxon, an irreversible AChE inhibitor (Eterovic et al., 2011). Here we show that, as predicted by the above-mentioned *in vitro* studies, 4R reduces brain damage caused by DFP.

Studying neurodegeneration and neuroprotection induced by administration of DFP to rats in doses near the LD_{50} is complicated by high mortality, caused by peripheral toxicity, and variable neurodegeneration in the survivors (Sparenborg et al., 1993; Crawford et al., 2004; Deshpande et al., 2010). Several peripherally acting drugs that do not cross the blood–brain barrier have been used to reduce mortality and at the same time allow for brain damage. We have chosen an experimental model in which pyridostigmine competes with DFP for the active site of AChE, protecting it from irreversible inhibition, while methyl atropine inhibits the overstimulation of muscarinic receptors (Kim et al., 1999; Li et al., 2011). At the beginning of these studies, methyl atropine was unavailable; therefore, we used the closest structural and functional analog, N-isopropyl atropine, or ipratropium, in three of the four experiments presented here.

2. Materials and methods

2.1. Drugs and reagents

Common laboratory chemicals and pyridostigmine bromide, ipratropium bromide monohydrate (N-isopropylatropine), 5,5-dithiobis(2-nitrobenzoic acid) (DTNB), acetylthiocholine iodide, sodium cacodylate, paraformaldehyde, xylene, potassium permanganate, dimethylsulfoxide, and diisopropylfluorophosphate were obtained from Sigma–Aldrich (St. Louis, MO). Methyl atropine nitrate was obtained from Spectrum Chemicals (New Brunswick, NJ), and Fluoro-Jade B was obtained from Millipore, (Billerica, MA, cat. no. AG310). The cembranoid (1S,2E,4R,6R,7E,11E)-cembra-2,7,11-triene-4,6-diol (4R) was prepared by Dr. K. El Sayed (School of Pharmacy, University of Louisiana, Monroe, LA) as previously described (El Sayed et al., 2008). The analytical criteria for characterization and purity were: 1) ^{1}H NMR: integration of the H-6 proton at d 4.81 *versus* that of the 4*S* epimer (d 4.46). 2) ^{13}C NMR: C-6 in the 4*R* (d_C 67.5) *versus* C-6 in the 4*S* (d_C 66.0), and 3) thin-layer chromatography (TLC): R_f value 0.42 (Si gel, *n*-hexane-ethyl acetate 1:1). The purity of the batch used for the present work was more than 98%.

2.2. Animals and treatments

Male Sprague-Dawley rats (240–315 g) from our colony derived from Taconic Farms were used. The animals were bred in the animal facility of Universidad Central del Caribe, Medical School. The animals were housed individually in positive-ventilation cages with filtered air (Allentown), controlled temperature and humidity, and a 12-h light–dark cycle. Food and water were provided *ad libitum*. All protocols were approved by the Institutional Animal Care and Use Committee of Universidad Central del Caribe.

The design of the experiments is illustrated in Fig. 2. The peripherally protective drugs, pyridostigmine bromide (0.1 mg/kg), ipratropium bromide (23 mg/kg), and methyl atropine nitrate (20 mg/kg) were dissolved in saline and injected *via* the i.m. route. DFP (9 mg/kg) was dissolved in cold water and injected i.p., while 4R (6 mg/kg) was dissolved in DMSO and injected s.c. in the interscapular area. The volume of injections was 1 ml/kg in all cases.

A series of experimental treatments comprising various combinations of drugs and times of 4R injection were tested. To describe each treatment in a succinct and unequivocal manner, we created the following system of nomenclature: 1) Each drug is abbreviated as follows: pyridostigmine (P), ipratropium (I), methyl atropine (M), dimethylsulfoxide (DMSO), diisopropylfluorophosphate (DFP), and 4R-cembranoid (4R). 2) The order of these abbreviations within the treatment name indicates the order in which the drugs were administered; *e.g.*, PI-DFP indicates that the animal received first P, then I, and finally DFP. 3) The time of 4R injection relative to DFP injection is indicated, *e.g.*, 1h4R-PI-DFP indicates that 4R was administered 1 h before DFP; 4R was then followed by the PI-DFP protocol as explained above.

2.3. Determination of acetylcholinesterase activity

AChE activity was measured as previously described (Ellman et al., 1961). The brains were dissected into the neocortex, hippocampus, striatum, remaining subcortex, cerebellum, and pons and medulla (Craigie et al., 1963). After dissection, the brain areas were weighed, frozen on dry ice, and stored at −80 °C. The tissue was homogenized in sodium phosphate buffer (0.1 M, pH 8.0 + 1% Triton X-100) at a concentration of 100 mg of wet weight per ml of buffer. The homogenates were centrifuged at 12,000 × *g* for 1 min, the supernatant collected, and tetraisopropyl pyrophosphoramide (100 μM) added to inhibit butyrylcholinesterase. AChE activity was measured in triplicate wells, and the color changes were read in a spectrophotometer at 405 nM with 16 kinetic cycles using a minimal kinetic interval. Enzyme activity was normalized to protein concentration, which was determined using Bradford reagent (Bradford, 1976). Data were expressed as μmoles of substrate transformed/min/mg of protein using the following formula:

$$\text{Activity} = \frac{(\Delta\text{OD/min sample} - \Delta\text{OD/min blank}) \times 0.2(\text{Total volume, ml})}{0.014(\text{Extinction cofficient, } \mu\text{M}^{-1}\ \text{cm}^{-1}) \times 0.01(\text{Sample volume, ml}) \times \text{Protein concentration(mg/ml)}}$$