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Carbamate nerve agent prophylatics exhibit distinct toxicological effects in the zebrafish embryo model

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ABSTRACT

Pyridostigmine bromide (PB) is an FDA-approved drug for the treatment of myasthenia gravis and a prophylactic pre-treatment for organophosphate nerve agent poisoning. Current methods for evaluating nerve agent treatments include enzymatic studies and mammalian models. Rapid whole animal screening tools for assessing the effects of nerve agent pre-treatment and post-exposure drugs represent an underdeveloped area of research. We used zebrafish as a model for acute and chronic developmental exposure to PB and two related carbamate acetylcholinesterase (AChE) inhibitors, neostigmine bromide (NB) and physostigmine (PS). Lethal doses and gross morphological phenotypes resulting from exposure to sub-lethal doses of these compounds were determined. Quantitative analyses of motility impairment and AChE enzyme inhibition were used to determine optimal dosing conditions for evaluation of the effects of carbamate exposures on neuronal development; ~50% impairment of response to startle stimuli and >50% inhibition of AChE activity were observed at 80 mM PB, 20 mM NB and 0.1 mM PS. PB induced stunted somite length, but no other phenotypic effects were observed. In contrast, NB and PS induced more severe phenotypic morphological defects than PB as well as neurite outgrowth mislocalization. Additionally, NB induced mislocalization of nicotinic acetylcholine receptors, resulting in impaired synapse formation. Taken together, these data suggest that altered patterns of neuronal connectivity contribute to the developmental neurotoxicity of carbamates and demonstrate the utility of the zebrafish model for distinguishing subtle structure-based differential effects of AChE inhibitors, which include nerve agents, pesticides and drugs.

1. Introduction

Acetylcholinesterase is a critical enzyme for synaptic transmission both between neurons at cholinergic synapses and across neuromuscular junctions (Soreq and Seidman, 2001). These activities underlie many of the essential biological functions, such as heartbeat, respiration, digestion, and brain activity. In addition to its established role in mature nervous systems, AChE may also have significant roles in neural system development, although these effects are not fully understood (Soreq and Seidman, 2001).

Several classes of small molecules modulate the activity of AChE – including organophosphate nerve agents and pesticides (Bajgar et al., 2009; Casida, 2009; Costa, 2006), carbamates (Bajgar et al., 2009) (Fig. 1A, B and C) and other small molecules (Bourne et al., 2004; Komloova et al., 2010), and they have varying effects (Grob, 1956). Organophosphates inhibit AChE through covalent modification of the active site serine with a phosphonate, phosphoamidate or phosphate moiety (Costa, 2006; Kropp and Richardson, 2007). Generally, organophosphate inhibition of AChE is irreversible, except when an AChE reactivator, such as 2-pralidoxime (2-PAM), is administered soon after exposure (Worek et al., 2013; Costa et al., 2011). In contrast, AChE can be readily reactivated following inhibition by a carbamate. Carbamates PB, NB and PS act on AChE by transfer of a carbamoyl group to the active site serine as illustrated in Fig. 1D. The resultant carbamoyl-serine is hydrolyzed by water with a half-life of several hours, depending upon

Abbreviations: α-btx, α-bungarotoxin; ACh, acetylcholine; AChE, acetylcholinesterase; AChRs, acetylcholine receptors; BBB, blood brain barrier; DMSO, dimethyl sulfoxide; dpf, days post fertilization; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); EM, embryonic media; GFP, green fluorescent protein; hpf, hours post fertilization; IC_{50}, dose resulting in 50% inhibition; LC_{50}, concentration lethal to 50% of animals tested; nAChR, nicotinic acetylcholine receptor; N, number of embryos tested per condition; NB, neostigmine bromide; PB, pyridostigmine bromide; PS, physostigmine; SLC, short latency C-start.

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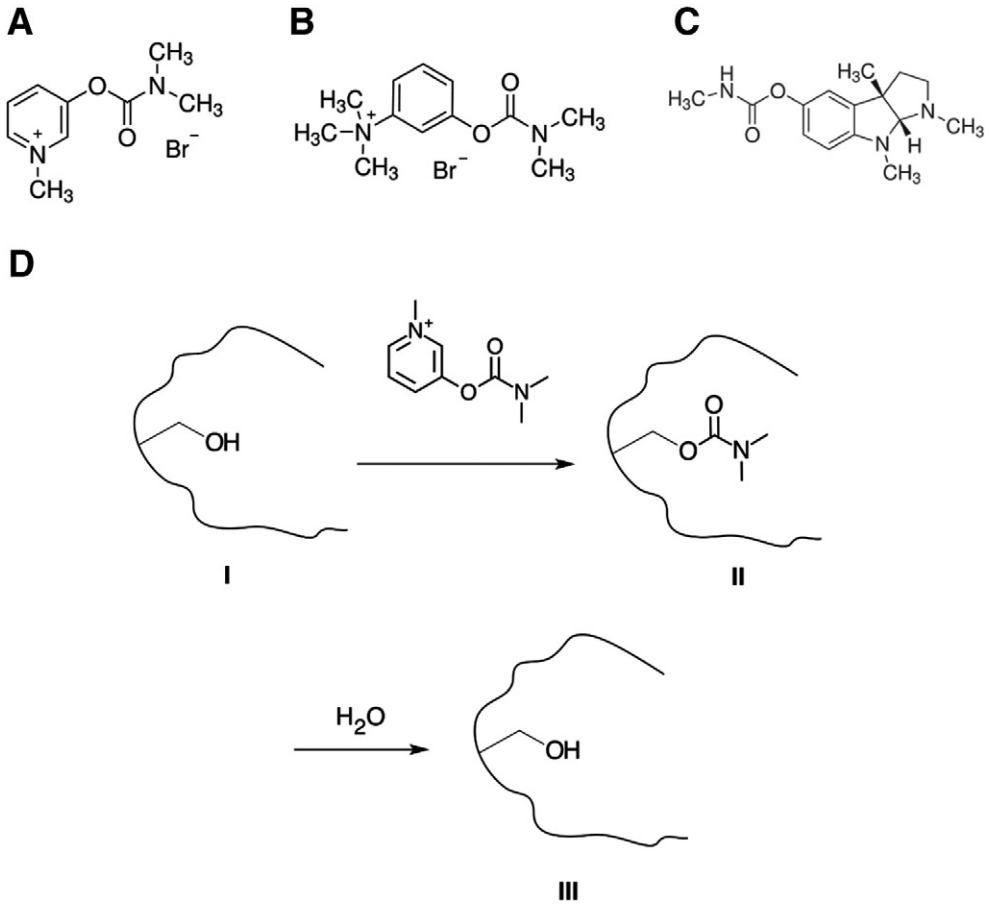

Fig. 1. Chemical structure of carbamate molecules investigated and mechanism of AChE inhibition. (A) PB is a pyridinium carbamate and an FDA-approved drug for prophylactic treatment of nerve agent exposure and for treatment of myasthenia gravis. (B) NB is a tetraalkylammonium carbamate, which is similar to PB in retaining a positive charge and is also used to treat myasthenia gravis. (C) PS is a natural product carbamate alkaloid, which is used as an antidote for the CNS effects of atropine, scopolamine and other anticholinergic overdoses. (D) Mechanism of AChE inhibition by PB. The active site Ser of AChE (I) attacks the carbamoyl carbon of PB, transferring the PB dimethylcarbamoyl group to the Ser hydroxyl (II). The inhibited AChE can be reactivated by hydrolysis (III).

the specific carbamate, and the decarbamylation rate can be increased by addition of an oxime (Harris et al., 1987; Dawson, 1995). After AChE reactivation, the degraded carbamate molecule is no longer capable of AChE inhibition. As such, the current prophylactic regimen for warfighters includes daily dosing with a carbamate (PB), which acts to hold a portion of the available AChE in reserve and protect it from inhibition by an organophosphate nerve agent. When a soldier is exposed to a nerve agent, he or she then self-administers a dose of 2-PAM via auto-injector that rapidly reactivates OP-bound AChE and speeds the reactivation of the carbamate-bound AChE reservoir, enabling a warfighter to survive the attack. The auto-injector also includes atropine, a muscarinic acetylcholine receptor antagonist, to mitigate the effects of cholinergic overstimulation that accompanies nerve agent exposure.

In addition to their utility as nerve agent prophylactics, carbamate drugs are also used as treatments for myasthenia gravis and Alzheimer's- and Parksinson's-related dementia (Van Der Putt et al., 2006; Imbimbo et al., 1999; De Sarno and Giacobini, 1989; Darreh-Shori and Jelic, 2010). Secondary targets of AChE inhibitors, such as other serine hydrolase enzymes (Vose et al., 2007; Quistad et al., 2006) and acetylcholine receptors (AChRs) (Silveira et al., 1990; Katz et al., 1997), have been implicated in the non-cholinergic effects of AChE inhibitors (De Sarno and Giacobini, 1989; Lotti and Moretto, 2006). In the case of pyridostigmine bromide (PB), secondary effects of this carbamate AChE inhibitor have been linked to the development of Gulf War Syndrome (Golomb, 2008a; Amourette et al., 2009), though these findings are still debated (Golomb, 2008b; Blazer et al., 2008). Even though many molecules in these families are commercially-used drugs or pesticides, there is still incomplete understanding of their mechanisms of action.

We aimed to develop a zebrafish model for rapid toxicological screening and mechanistic investigation of carbamate AChE inhibitors as a first step toward developing rapid whole-animal protocols for evaluation the drug combinations used to treat nerve agent poisoning. The zebrafish (*Danio rerio*) has been used to investigate chemicals affecting synapse-associated enzymes and receptors (Hanneman and Westerfield, 1989; Behra et al., 2002, 2004). As a model organism, zebrafish have a number of advantages relative to mammaliam models. A key feature of zebrafish for the study of the cholinergic system is that AChE is the only known ACh-hydrolyzing enzyme present in the organism (Hanneman and Westerfield, 1989). In addition, carboxylesterase activity is minimally affected by PB and related carbamates (Shaikh and Pope, 2003; Maxwell et al., 1988; Gupta and Dettbarn, 1993). Zebrafish embryos have rapid ex utero development and can be generated in large quantities for high-throughput experiments. The embryos maintain optical clarity through 5 days post fertilization (dpf), which facilitates imaging studies. Additionally, a large number of transgenic lines are available for mutational studies.

Zebrafish have been previously used for evaluation of neuro-effective small molecules. Chlorpyrifos, an organophosphate pesticide and irreversible AChE inhibitor, elicits neurobehavioral effects and corresponding impairment of sensory neuron development in zebrafish (Levin et al., 2003; Jacobson et al., 2010). Treatment of zebrafish with edrophonium or tacrine, both FDA-approved drugs and reversible AChE inhibitors, causes sensory neuron mislocalization (Behra et al.,