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Original paper

Detoxification of tabun-exposed mice by an acetylcholinesterase mutant using a novel pyridinium aldoxime

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Summary. Nerve agents, such as tabun, are covalent inhibitors of acetylcholinesterase (AChE), an essential enzyme in neurotransmission whose inhibition may lead to death. Currently used therapy, consisting of an anticholinergic drug and an oxime as the reactivator of inhibited AChE is particularly ineffective in cases of tabun exposure. Thus, an optimal re-activator is still needed. Click-chemistry, utilizing Cu (I)-catalyzed azide-alkyne cycloaddition of a library of small molecule building blocks, has made possible the rapid synthesis of a variety of new oximes. Among the new oximes tested in recent studies as re-activators of tabun-inhibited choline binding site AChE mutants, oxime **5B**, a lengthened alkylchain congener of the standard oxime 2-PAM, as appeared to be a candidate for tabun *ex vivo* scavenging when paired with the Y337A mutant of AChE. Herein, we pursued the antidotal *in vivo* detoxification of tabun-exposed mice by assembling oxime-assisted catalytic scavenging using the mutant combined with oxime **5B.** Although the antidotal treatment requires optimization, our findings offer a platform for further development of more potent means of counteracting tabun and related phosphoramidate exposure.

Keywords: antidotes, bioscavengers, cholinesterase, nerve agents, organophosphorus compounds, 2PAM.

INTRODUCTION

The high toxicity of nerve agents arises from irreversible inhibition of acetylcholinesterase (AChE; EC 3.1.1.7), an enzyme with an essential role in neurotransmission. Inhibition of AChE activity results in the accumulation of the neurotransmitter acetylcholine at vital cholinergic sites, which in turn leads to life-threatening toxic manifestations. Currently used therapies rely on an anticholinergic (atropine) and oximes as re-activators of inhibited AChE (Gray 1984; Dawson 1994). However, this approach has limitations, mostly due to the inability of the applied standard oximes (TMB-4, obidoxime, HI-6 and 2-PAM) to efficiently reactivate AChE inhibited by various organophosphates (OP) (Jokanović and Prostan 2009). Moreover, therapy is particularly ineffective in cases of tabun exposure due to the presence of an electron pair located on the amidic group of tabun, which makes nucleophilic attack by an oxime unfavorable (Eto 1976; Artursson et al. 2009; Katalinić et al. 2018). In addition, formation of the AChE-tabun conjugate leads to the creation of steric hindrance in the active center gorge preventing oxime-assisted reactivation (Ekstrom et al. 2006a, 2006b). Therefore, other modes of inhibition of tabun bioactivity are being intensively studied (Huang et al. 2007; Kovarik et al. 2007a, 2009; Lenz et al. 2007; Artursson et al. 2009; Cohran et al. 2011; Radić et al. 2013; Masson and Nachon 2017; Katalinić et al. 2018).

One approach suggests employing an enzyme scavenger and an oxime re-activator as a pair capable of rapidly neutralizing tabun, in cycles, before it reaches targeted synaptic AChE. It has been proven that site-directed mutagenesis could be used to optimize reactivation of phosphorylated

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AChE assisted by oximes (Kovarik et al. 2004, 2007b; Artursson et al. 2009; Katalinić and Kovarik 2012; Kovarik et al. 2013). Along with endogenous butyrylcholinesterase (BChE) (Lucić Vrdoljak et al. 2006; Kovarik et al. 2010; Radić et al. 2013, Vučinić et al. 2013; Katalinić et al. 2016), AChE mutants could be considered as bioscavengers of nerve agents. Our recent studies reported the first *in vivo* example of effective oxime-assisted catalytic soman and VX bio-scavenging based on the combined administration of substoichiometric amounts of the choline-binding site AChE mutant (Y337A/F338A) and oxime HI-6 (Kovarik et al. 2015; Maček Hrvat et al. 2016).

Recently we reported several novel oximes with a triazole ring synthesized using copper-catalyzed azide-alkyne cycloaddition click chemistry reaction that were particularly efficient re-activators in tabun-inhibited AChE (Kovarik et al. 2019a). After initial screening of the triazole oxime library and its precursors for reactivation efficacy on Y337A and Y337A/F338A human AChE mutants, we found potentially active oxime-mutant enzyme pairs capable of degrading tabun in cycles of inhibition and reactivation (Kovarik et al. 2019b). Surprisingly, the most promising *ex vivo* reactivation of the mutant occurred with alkylpyridinium aldoximes. In this study, to validate the *in vitro* and *ex vivo* obtained results (Kovarik et al. 2019a, 2019b), and examine the potential for further development of bioscavengers using AChE mutants, as well as identify any potential problems, a lead oxime-enzyme pair was tested *in vivo* on tabun-exposed mice.

MATERIALS AND METHODS

Chemicals and enzymes

1-Hexyl-2-((hydroxyimino)methyl)pyridinium chloride (5B) was prepared as described previously (Kovarik et al. 2019a), and was generously provided by Prof K. Barry Sharpless (Skaggs Institute for Chemical Biology, The Scripps Research Institute, La Jolla, USA). Atropine sulphate was purchased from Kemika, Zagreb, Croatia. A solution of 5 mg/mL of atropine was prepared in distilled water. The oxime was dissolved in atropine or in distilled water just before use. Tabun [ethyl N,N-dimethylphosphoroamidocyanidate] was purchased from NC Laboratory, Spiez, Switzerland. A stock solution of 5000 μg/mL of tabun was prepared in isopropyl alcohol. Further dilutions were made in saline, shortly before use. Recombinant DNA-expressed human AChE mutant Y337A was prepared as described in Cochran et al. (2011), and was a generous gift from Prof Palmer Taylor (Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California, San Diego, La Jolla, California, USA). The enzyme (0.5 mg/mL) was dissolved in saline just before use.

Detoxification of tabun in exposed mice

Male CD-1 mice (35–40 g body weight; purchased from Ruđer Bošković Institute, Zagreb, Croatia) were treated with the approval of the Ethics Committee of the Institute for Medical Research and Occupational Health in Zagreb, Croatia. For experiments, mice were divided into groups of four.

Antidotal efficacy of oxime **5B** against tabun poisoning was tested by administering 40 mg/kg oxime (dose equals to 25% of its LD_{50}; no toxic signs were observed) intramuscularly together with atropine sulphate (10 mg/kg) 1 min after subcutaneous tabun exposure in the mice. To test the catalytic detoxification of tabun-exposed mice, a combination of pretreatment and therapy was applied. Mice were pretreated intravenously with human AChE mutant Y337A (1 mg/kg) 15 min prior to tabun exposure. All mice were treated intramuscularly with **5B** (40 mg/kg) and atropine (10 mg/kg) 1 min after tabun exposure. Four mice received a second dose of **5B** (40 mg/kg) 30 min after the first one. Symptoms of poisoning were monitored up to 24 h.

Acute toxicity (LD_{50}) was based upon 24 h mortality rates and was estimated from tables described elsewhere (Thompson et al. 1947; Weil et al. 1952). The antidotal efficacy was expressed as a protective index (PI) with 95% confidence limits and maximal dose of tabun (MD). The PI is the ratio of LD_{50} between tabun with treatment and tabun administered alone, without treatment. The MD is the highest multiple of the tabun LD_{50}, which was fully counteracted by the treatments.

RESULTS AND DISCUSSION

In our recent studies, mutant AChE and oxime HI-6 were administrated to soman- or VX-poisoned mice; results suggest that catalytic bio-scavenging improved therapeutic outcomes preventing lethality and delaying the onset of toxicity symptoms (Kovarik et al. 2015; Maček Hrvat et al. 2016). Therefore, based on *in vitro* results and the recently reported e*x vivo* detoxification of tabun-exposed human whole blood (Kovarik et al. 2019a, 2019b), we selected the human AChE mutant Y337A and the pyridinium oxime **5B** for further tests in mice. The aim was to perform detoxification of tabun by assembling oxime-assisted catalytic scavenging *in vivo* using the AChE mutant Y337A and an efficient re-activator.

The determined acute toxicity of several doses of **5B** oxime for mice was 158.8 and 10 mg/kg for *i.m*. and *i.v.*, respectively, classifying **5B** as a relatively toxic compound especially for intravenous application. Therefore, in this detoxification study we administrated oxime *i.m.* as post exposure treatment and enzyme *i.v.* as pretreatment. No toxicity symptoms were observed upon *i.v.* administration of human AChE mutant Y337A (1 mg/kg). The antidotal efficacy of

Table 1. Antidotal and scavenging efficacy of oxime 5B alone and together with human AChE Y337A mutant in tabun-exposed mice. The results are presented as protective index, PI (95% confident limits are given in parentheses) and maximal dose of tabun, MD.

Treatment (time of application)[a]	PI	MD
Tabun (0') + 5B with atropine (1')	1.6 (1.2 – 2.1)	1
Y337A (0') + tabun (15') + 5B with atropine (16')	n.d.[b]	<1
Y337A (0') + tabun (15') + 5B with atropine (16') + 5B (46')	1.4 (1.1 – 1.7)	1

[a]All of the groups received oxime 5B (40 mg/kg equals 0.25 of its LD_{50}; *i.m.*) in atropine (10 mg/kg) 1 min after tabun exposure (*s.c.*). One group received additional dose of 5B (40 mg/kg; *i.m.*) 30 min after the first administration. Two groups were pretreated with human AChE mutant Y337A (1 mg/kg, *i.v*) 15 min prior to tabun exposure (*s.c.*). LD_{50} (*s.c.*) of tabun was 267 μg/kg. The experiment utilized four mice in each group.
[b] n.d.-not determined.

the tested scavenging system in terms of protective index (PI) and maximal dose of tabun (MD) is given in Table 1. The post exposure therapy with 5B alone ensured a 1.6-fold protection of mice, and all of the mice survived a dose of 267 μg/kg of tabun. However, pretreatment of mice with Y337A mutant alone did not improve protection. Similarly, repetition of the oxime dose (*i.m.*) 30 min after the first dose only slightly reduced the symptoms of poisoning (Fig. 1), but did not influence lethality outcomes (Fig. 2).

In summary, no significant protection of tabun-exposed mice was observed. The failure of the Y337A mutant and re-activator **5B** to detoxify tabun in mice could be a consequence of many factors such as: oxime toxicity, low application dose of oxime, use of two routes for therapy administration, species differences, mouse *vs.* human, etc. It should also be pointed out that oxime **5B** showed poor potency for reactivation of phosphorylated endogenous AChE and BChE as reported previously (Kovarik et al. 2019b) and presented in Figure 2. More precisely, to be efficient in the overall combined treatment as applied in the present study, an oxime alone should not only reactivate exogenous enzyme but also endogenous erythrocyte AChE and plasma BChE. This is in accord with our previous studies with potent re-activators of tabun-inhibited AChE, where we reported that the therapeutic efficacy *in vivo* corresponded to reactivating efficacy *in vitro*, meaning that the pharmacological effect of these oximes was indeed primarily related to the reactivation of tabun-inhibited AChE (Čalić et al. 2006, 2008; Kovarik et al. 2008, 2009; Berend et al. 2010). Moreover, this was confirmed with the best triazole oxime re-activators of the AChE wild type, where the protection index was 8.9, and all of the mice survived a dose of 7.9 LD_{50} of tabun (Kovarik et al. 2019a) despite the relatively high toxicity of the lead

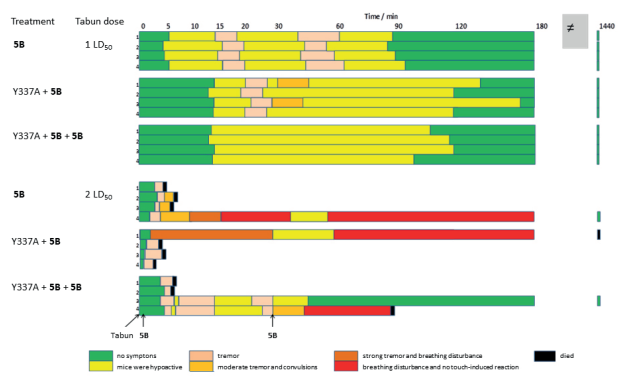

Fig. 1. Severity of toxicity symptoms in mice exposed to tabun (s.c.). All of the groups received oxime **5B** (40 mg/kg equals 0.25 of its LD_{50}; *i.m.*) in atropine (10 mg/kg) 1 min after tabun exposure (*s.c.*). One group received additional dose of **5B** (40 mg/kg; *i.m.*) 30 min after the first administration. Two groups were pretreated with human AChE mutant Y337A (1 mg/kg, *i.v*) 15 min prior to tabun exposure (*s.c.*). LD_{50} (*s.c.*) of tabun was 267 μg/kg. The experiment utilized four mice in each group.

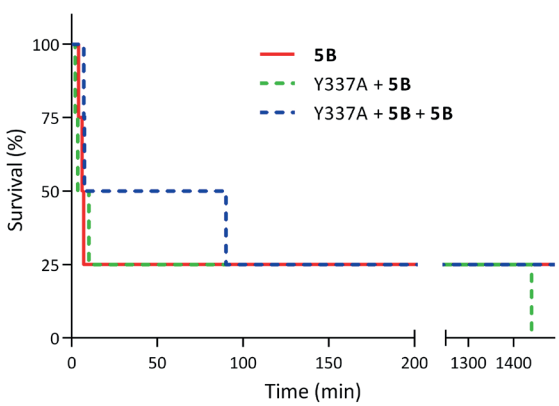

Fig. 2. Survival plots for mice exposed to 2 x LD_{50} of tabun (*s.c.*). All of the groups received oxime **5B** (40 mg/kg equals 0.25 of its LD_{50}; *i.m.*) in atropine (10 mg/kg) 1 min after tabun exposure (*s.c.*). One group received additional dose of **5B** (40 mg/kg; *i.m.*) 30 min after the first administration. Two groups were pretreated with human AChE mutant Y337A (1 mg/kg, *i.v*) 15 min prior to tabun exposure (*s.c.*). LD_{50} (*s.c.*) of tabun was 267 μg/kg. The experiment utilized four mice in each group.

oxime and the low dose (2.2 mg/kg oxime).

Furthermore, extrapolation of data obtained *in vitro* to *in vivo* applications could hinder efficient therapy. Specifically, although oxime **5B** has a similar pK_a (Radić et al. 2013; Kovarik et al. 2019b), and non-cytotoxic profile *in vitro* (Kovarik et al. 2019b) compared with standard oximes 2-PAM and HI-6, antidotal therapy is limited by oxime circulation and tissue-specific distribution (Katalinić et al. 2015; Sit et al. 2018), and therefore the applied dose becomes important. When we administrated non-toxic oxime HI-6 intravenously together with the mutant to poisoned mice, soman and VX were efficiently detoxified (Kovarik et al. 2015; Maček Hrvat et al. 2016).

In conclusion, although co-treatment with a Y337A mutant and re-activator **5B** has specific shortcomings, our findings offer a platform for further development of more potent means of counteracting tabun and related phosphoramidate exposure.

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Conflict of interest: The authors declare that they have no conflict of interest.

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