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Restoration of soman-blocked neuromuscular transmission in human and rat muscle by the bispyridinium non-oxime MB327 *in vitro*

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ABSTRACT

The standard treatment of poisoning by organophosphorus (OP) nerve agents with atropine and oximes is not sufficiently effective against all types of nerve agents. Alternative therapeutic strategies are required and bispyridinium non-oximes, acting as nicotinic antagonists, were identified as promising compounds. A previous study showed that the di(methanesulfonate) salt of the bispyridinium compound MB327 could restore soman-impaired neuromuscular function *in vitro* and improve survival of sarin, soman and tabun poisoned guinea pigs *in vivo*. Here, by using the indirect field stimulation technique, the ability of MB327 to counteract soman-impaired neuromuscular transmission was investigated in human intercostal muscle and rat diaphragm preparations. MB327 restored muscle force in a concentration-dependent manner in both species without reactivating soman-inhibited acetylcholinesterase. The therapeutic effect of MB327 could be washed out, indicating a direct effect at the nicotinic receptor level. Also the ability of MB327 to restore respiratory muscle function could be demonstrated for the first time in rat and human tissue. In combination with previous *in vitro* and *in vivo* findings MB327 may be considered a promising compound for the treatment of nerve agent poisoning and further studies are needed to identify optimized drug combinations, concentrations and dosing intervals to provide an effective therapy for OP poisoning.

1. Introduction

Despite on-going efforts of the international community to abandon chemical warfare agents, highly toxic organophosphorus (OP) nerve agents continue to pose a serious threat to military personnel and the civilian population (Wiener and Hoffman, 2004). The primary mechanism of action of OP nerve agents is the irreversible inhibition of the pivotal enzyme acetylcholinesterase (AChE), resulting in an accumulation of the neurotransmitter acetylcholine at cholinergic synapses followed by disturbance of numerous body functions and finally death due to respiratory failure (Holmstedt, 1959; Sidell, 1997).

Basic treatment of OP poisoning with atropine is effective to counteract the toxic effects symptomatically at muscarinic receptors but has no therapeutic effect at nicotinic receptors (McDonough and Shih, 2007). Hence, atropine needs to be supplemented by oximes able to reactivate OP-inhibited AChE and to antagonize the OP effects in target organs that are mediated by nicotinic receptors (Eyer and Worek, 2007). This therapeutic approach is of utmost importance for the recovery of OP-impaired respiratory muscle function and a prerequisite for survival of OP poisoned patients.

However, clinically used (*e.g.* obidoxime, pralidoxime) as well as experimental oximes (*e.g.* HI-6) have a limited therapeutic efficacy in cases of poisoning by different nerve agents (*e.g.* soman, tabun) (Eyer and Worek, 2007; Worek et al., 2004; Bismuth et al., 1992). The underlying mechanism may be either rapid aging of the inhibited enzyme in the case of soman or the formation of a reactivation-resistant AChE-OP-complex in case of tabun (Worek et al., 2004, 2007a). Recently, an *in vitro* study with human intercostal muscle preparations demonstrated the inability of supra-therapeutic HI-6 concentrations (up to 1000 μM) to restore soman-impaired neuromuscular transmission (Seeger et al., 2011).

Hence, there is an urgent need to develop alternative therapeutic means in order to improve treatment of poisoning by nerve agents. Recent studies provided promising *in vitro* results

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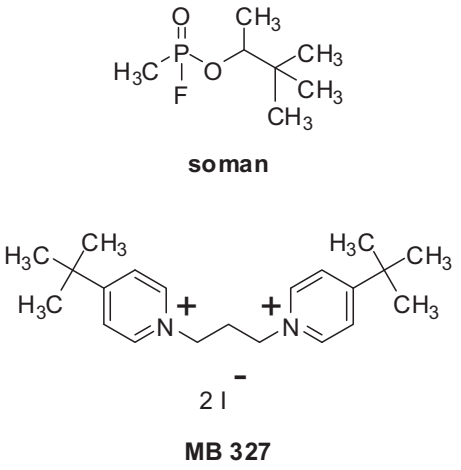

Fig. 1. Chemical structures of soman and MB327.

with bispyridinium non-oximes (Tattersall, 1993; Turner et al., 2011), in which the di(methanesulfonate) salt of the bispyridinium compound MB327 (Fig. 1) demonstrated its efficacy in restoring soman-impaired neuromuscular transmission of guinea-pig diaphragms, improving survival in sarin, soman and tabun poisoned guinea-pigs (Timperley et al., 2011; Turner et al., 2011). We have now evaluated the potential therapeutic effect of MB327 in an *in vitro* model for testing neuromuscular function with muscle samples from different species, including man. By utilizing an established indirect field stimulation technology (Seeger et al., 2007, 2011) we were able to investigate for the first time the effect of MB327 on soman-impaired human intercostal muscle function.

2. Materials and methods

2.1. Chemicals

Soman (>98% by GC–MS, ^{1}H and ^{31}P NMR; Fig. 1) was made available by the German Ministry of Defence. MB 327 (>98%; Fig. 1) was synthesized at Dstl Porton Down, UK, as described before (Timperley et al., 2011). Pancuronium bromide, Triton X-100 and acetylcholine iodide were purchased from Sigma Aldrich (Taufkirchen, Germany). [^{3}H] acetylcholine iodide (2 GBq/mmol, 97% radiochemical purity), OptiScint HighSafe lipophilic scintillation cocktail and polyethylene Pico Prias vials were obtained from Perkin Elmer (Rodgau-Jügisheim, Germany). Aqua Safe 300 Plus was supplied by Zinsser Analytic (Frankfurt/Main, Germany). BC Assay protein quantification kit (Uptima-Interchim) and all other chemicals of the purest grade available were purchased from E. Merck (Darmstadt, Germany).

2.2. Human muscle preparation

Intercostal muscle biopsies (approx. size: 5 cm × 1.5 cm, thickness: 0.5 cm) were taken from 7 patients (age 67.7 ± 8.2 years) undergoing therapeutic cancer surgery (lung carcinoma). The Ethics Committee of the Ludwig-Maximilians-University, Munich, approved the study protocol and the patients gave their written informed consent.

The muscle biopsies were immediately placed on cold (4 °C) 0.9% NaCl moist gauze and were transported to the Bundeswehr Institute of Pharmacology and Toxicology within 1 h. In the laboratory the muscle biopsies were immediately placed into gassed (95% O_2 and 5% CO_2) Tyrode solution (125 mM NaCl, 24 mM $NaHCO_3$, 5.4 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 2.5% glucose) (Bülbring, 1946; Taugner et al., 1950). Adequate samples (0.37 ± 0.24 g; approx. size: 2.1 cm × 0.9 cm, thickness: 0.5 cm) were prepared, transferred to a horizontal organ bath and circumfused with Tyrode solution (8 mL/min, 25 ± 0.5 °C) to avoid O_2 and glucose deprivation of the inner fibers (Segal and Faulkner, 1985). One side of the muscle was attached to a fixed hook, the other one to a force transducer (HSE Force Transducer F-30). With a pair of platinum electrodes, placed parallel to the muscle fibers, electric field stimulation was performed with rectangular pulses of 5 μs width and amplitudes of 0.35–0.4 A. Tetanic trains with a duration of 2 s with frequencies of 25 and 40 Hz were applied at 10 s intervals (Seeger et al., 2007). Isometric muscle force production was measured and the amplified signals were recorded online with a sample frequency of 1 kHz (CellWorks software, npi electronic, Tamm, Germany).

2.3. Rat diaphragm preparation

Male Wistar rats (300 g ± 50 g, Charles River, Sulzfeld, Germany) were anesthetized with CO_2 and decerebrated. Hemidiaphragm preparations were rapidly excised, mounted vertically in glass chambers (high throughput myograph system with 12 chambers) and secured to force transducers (World Precision Instruments, Sarasota, FL, USA) (Seeger et al., 2007). The muscles were incubated with aerated Tyrode solution (25 ± 0.5 °C). Electric field stimulation was performed with a pulse width of 50 μs and amplitude of 0.2 A. Tetanic trains with frequencies of 25, 50 and 100 Hz for 1 s in 5 s intervals were applied. Isometric muscle force production was determined every 5 min. The amplified signals were recorded online with a sample frequency of 1 kHz (ACQ software, Biopac Systems, Goleta, USA). Tyrode solution was changed every 30 min. All procedures using animals followed animal care regulations and were approved by the responsible ethics committee.

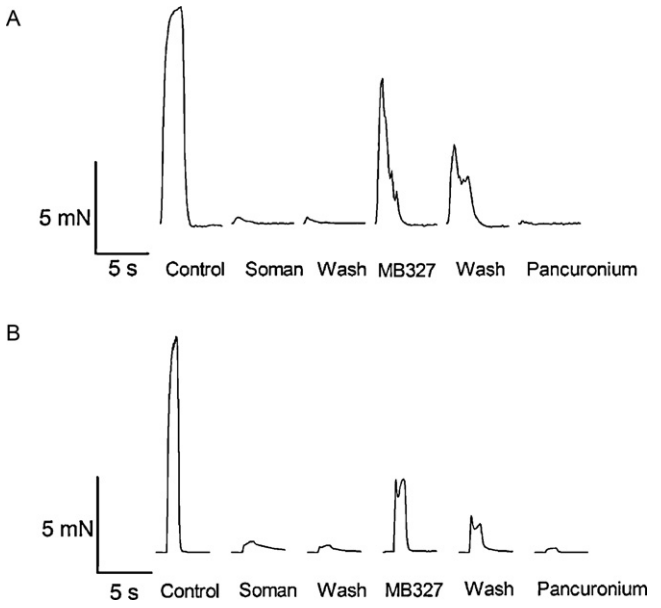

Fig. 2. Effect of soman (3 μM) and MB327 (100 μM) on muscle force production after indirect stimulation. (A) Representative recordings of muscle force generation with a human intercostal muscle preparation stimulated for 2 s at 25 Hz. (B) Representative recordings of muscle force generation with a rat diaphragm preparation stimulated for 1 s at 25 Hz.

2.4. Experimental protocol

Following a 60 min equilibration with plain Tyrode solution, a test sequence was applied to obtain maximal muscle force generation (control = 100%; Fig. 2). Thereafter, the muscle preparations were exposed to 3 μM soman for 30 min and muscle force generation was recorded every 10 min. Following a 30 min wash-out period MB327 was added. Rat hemidiaphragm preparations were incubated with cumulative concentrations of MB327 (1–300 μM, 30 min each). With human muscle samples, MB327 at 100 or 200 μM (30 min each) was added to separate preparations. The effect of MB327 was recorded in the presence of the test compound and after 30 min wash-out. Finally, force production was measured in presence of 1 μM pancuronium bromide to confirm that the electrical field stimulation technique induced muscle contraction *via* neuromuscular endplates. Thereafter, the preparations were frozen and stored at −80 °C until analysis of AChE activity within 1 month.

2.5. Determination of AChE activity

The muscle tissue samples were homogenized as described before (Thiermann et al., 2005). In brief, muscle tissue was separated from connective tissue and was homogenized on ice in Tris buffer (50 mM Tris–HCl, 1 M NaCl, 1% Triton-X 100, pH 7.4) at a ratio of 100 mg tissue/mL buffer with an Ultra Turrax T 25 disperser (Janke & Kunkel, Staufen, Germany, 19.000 rpm, 50 s). The suspension was further homogenized on ice with a glass-Teflon potter at a ratio of 50 mg tissue/mL buffer (PotterS, Braun, Melsungen, Germany, 1050 rpm, five up and down strokes, 10 s). Muscle homogenate aliquots were stored at −80 °C until further use. AChE activity was determined by a radiometric assay as described before (Thiermann et al., 2005). The protein concentration was determined with the bicinchoninic acid assay (BCA) method (Smith et al., 1985) using bovine serum albumin for external standard calibration.

2.6. Data analysis

Muscle force was analyzed as absolute force generation or as a time–force integral (area under the curve, AUC). Results are expressed as a percentage of the control. AChE activity is given as U/mg protein. All data are shown as means ± SD. One-way analysis of variance (ANOVA) with Bonferroni post-test was employed to