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Pharmacokinetic profile and quantitation of protection against soman poisoning by the antinicotinic compound MB327 in the guinea-pig

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HIGHLIGHTS

- The bispyridinium compound MB327 protects guinea-pigs from soman poisoning.
- Mode of action is not reliant on reactivation of aged inhibited acetylcholinesterase.
- First syntheses of d6-MB327 diiodide and dimethanesulfonate salts.
- Used as internal standards for mass spectrometric quantitation of MB327 in guinea-pig plasma.

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ABSTRACT

Current organophosphorus nerve agent medical countermeasures do not directly address the nicotinic effects of poisoning. A series of antinicotinic bispyridinium compounds has been synthesized in our laboratory and screened in vitro. Their actions can include open-channel block at the nicotinic receptor which may contribute to their efficacy. The current lead compound from these studies, MB327 1,1′-(propane-1,3-diyl)bis(4-*tert*-butylpyridinium) as either the diiodide (I_2) or dimethanesulfonate (DMS) has been examined in vivo for efficacy against nerve agent poisoning. MB327 I_2 (0–113 mg kg^{-1}) or the oxime HI-6 DMS (0–100 mg kg^{-1}), in combination with atropine and avizafone (each at 3 mg kg^{-1}) was administered to guinea-pigs 1 min following soman poisoning. Treatment increased the LD_{50} of soman in a dose-dependent manner. The increase was statistically significant ($p < 0.01$) at the 33.9 mg kg^{-1} (MB327) or 30 mg kg^{-1} (HI-6) dose with a comparable degree of protection obtained for both compounds. Following administration of 10 mg kg^{-1} (i.m.), MB327 DMS reached plasma C_{max} of 22 μM at 12 min with an elimination $t_{1/2}$ of 22 min. In an adverse effect study, in the absence of nerve agent poisoning, a dose of 100 mg kg^{-1} or higher of MB327 DMS was lethal to the guinea-pigs. A lower dose of MB327 DMS (30 mg kg^{-1}) caused flaccid paralysis accompanied by respiratory impairment. Respiration normalised by 30 min, although the animals remained incapacitated to 4 h. MB327 or related compounds may be of utility in treatment of nerve agent poisoning as a component of therapy with atropine, anticonvulsant and oxime, or alternatively as an infusion under medical supervision.

1. Introduction

Medical countermeasures (MedCMs) to nerve agent poisoning do not currently contain a component which directly counteracts the effect of elevated acetylcholine at nicotinic synapses. While oximes alleviate this by restoring acetylcholinesterase (AChE) function at these sites (Timperley et al., 2011), there are limitations to the efficacy of oximes for AChE inhibited by some nerve agents such as tabun (Heilbronn and Tolagen, 1965) or where rapid 'ageing' of the inhibited enzyme occurs such as with the AChE-soman adduct (Fleisher et al., 1965). A series of bispyridinium compounds has been synthesized and screened for ion-channel blocking properties at the nicotinic receptor (Timperley et al., 2005). In vitro studies have demonstrated that one of these

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compounds, MB327, restores function in nerve agent-poisoned guinea-pig, rat and human muscle preparations (Seeger et al., 2012; Turner et al., 2011). Related compounds also bind to the human alpha 7 nicotinic acetylcholine receptor and the nicotinic receptors from electric eel *Torpedo californica* (Niessen et al., 2012a, 2011). MB327 binds to the human M_5 muscarinic acetylcholine receptor (Niessen et al., 2012b) and causes a relaxation of rat jejunum smooth muscle (Königer et al., 2013).

MB327 (Fig. 1) has been assessed in vivo in mouse and guinea-pig models of nerve agent poisoning. In atropine-pretreated mice, MB327 (referred to as P62) provided some protection, reducing an LD_{95} dose of soman to an LD_{50} (Schoene et al., 1976). In guinea-pigs, MB327 DMS, in combination with physostigmine salicylate and hyoscine hydrobromide, protected 4/8 of animals against a $5 \times LD_{50}$ challenge of sarin and 5/8 against $5 \times LD_{50}$ of tabun. In comparison, HI-6 protected 8/8 and 1/8 animals, respectively (Turner et al., 2011). In the same model, protection against soman provided by MB327 DMS was 6/8, and by HI-6, 7/8 (Timperley et al., 2012). This broadly similar efficacy across nerve agents with different oxime sensitivities and ageing rates suggests MB327 and related bispyridinium compounds may provide a broad-spectrum adjunct to conventional therapy for nerve agent poisoning. However, these previous studies did not use the additional therapy components used in current MedCMs (antimuscarinic and anticonvulsant) (Timperley and Tattersall, 2015). An assessment was therefore conducted to determine if MB327 was beneficial when administered in combination with the currently-fielded UK MedCM combination of atropine sulfate and avizafone hydrochloride. A comparison was also made to a combination treatment comprised of atropine sulfate, avizafone and the oxime HI-6. Soman was selected as the challenge agent for these studies as soman-inhibited AChE ages rapidly rendering it resistant to oxime reactivation. Poisoning by soman thus poses one of the more difficult challenges for MedCM.

Both MB327 I_2 and MB327 DMS were studied. Initially, the former was available in greater quantities than the latter, which is synthesised from the diiodide by ion exchange (Timperley et al., 2012). The decision was made to use MB327 I_2 for efficacy studies, adjusting its concentration to account for the different molecular masses of MB327 I_2 and MB327 DMS, and to save the latter for the pharmacokinetic (PK) studies. The dimethanesulfonate salt is preferable for PK studies due to its greater water solubility. Syntheses of comparable salts with a d_6-deuterated linker and their use as internal standards for mass spectrometric quantitation of MB327 I_2 and MB327 DMS in guinea-pig plasma are also described.

2. Methods

2.1. Animals

All animal work was carried out under the terms and conditions of a Project Licence (PPL 30/2864) granted by the UK Home Office under the Animals (Scientific Procedures) Act, 1986. Male HsdDhl:DH Dunkin–Hartley guinea-pigs were supplied by Harlan Interfauna (Hillcrest, UK). Bodyweight on arrival was 233 ± 9 g (n = 76; mean ± SD) for the efficacy study, 245 ± 6.4 g (n = 30; mean ± SD) for the adverse events study and 334 ± 31 g (n = 7; mean ± SD) for the PK study. The animals were allowed to acclimatize for at least 4 days prior to study, and were kept in standardised conditions to meet UK Home Office guidelines (room temperature 21 °C, humidity 50%). The lights were on from 06:00 to 18:00 h. Daily body weight and temperature recordings were monitored throughout the experiment as a measure of health and wellbeing.

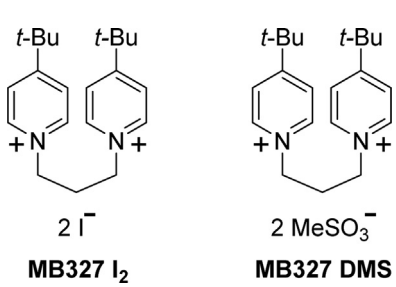

Fig. 1. Antinicotinic bispyridinium compounds.

2.2. Chemicals and drugs

Atropine sulfate was supplied by Sigma Ltd. (Dorset, UK), avizafone by Roche Ltd. (Welwyn Garden City, UK), HI-6 by Phoenix Chemicals Ltd. (Bromborough, UK) and soman by chemists working in the UK Single Small Scale Facility, Dstl Porton Down (>95% pure, diluted in isopropanol). d_6-1,3-Dibromopropane and other reagents were from Aldrich Ltd. (Dorset, UK) and were used as received. Thin layer chromatography (TLC) was performed on MK6F silica gel 60 Å plates (Whatman, USA) with detection by iodine vapour. Nuclear magnetic resonance (NMR) data were collected at 9.4 T using a Bruker Avance III spectrometer equipped with a 5 mm BBFO + probehead.

2.3. Deuterated standards

Undeuterated MB327 I_2 and MB327 DMS were prepared according to a published procedure (Timperley et al., 2012). Deuterated analogues were synthesized by Finkelstein reaction of d_6-1,3-dibromopropane with an excess of sodium iodide in acetone (Fig. 2). The d_6-1,3-diiodopropane (1) obtained was bis-quaternized by two molar equivalents of 4-*tert*-butylpyridine in boiling nitromethane to give d_6-MB327 I_2. Treatment of this with a suspension of silver(I) methanesulfonate in methanol provided d_6-MB327 DMS.

2.3.1. d_6-1,3-Diiodopropane (1)

A solution of d_6-1,3-dibromopropane (5.0 g, 24 mmol) in acetone (15 ml) was added dropwise over 20 min to a stirred solution of sodium iodide (15.0 g, 100 mmol) in acetone (55 ml) in a 250-ml round-bottomed flask. The addition caused precipitation of sodium bromide. Stirring was continued for 12 h. The sodium bromide was filtered off and rinsed thoroughly with acetone. The solvent was removed from the filtrate to leave sludge. This was partitioned between dichloromethane (55 ml) and water (30 ml) and the aqueous layer extracted with dichloromethane (2 × 30 ml). The organic layers were combined and dried ($MgSO_4$), the drying agent filtered off, and the solvent removed from the filtrate to leave a mobile yellow liquid (8.56 g). Fractionation of this using a micro-distillation apparatus gave the title compound as a pale orange mobile liquid (6.38 g, 88%). Bp 61 °C/1 mmHg. Analysis by gas chromatography–mass spectrometry showed that it was 99% pure (the 1% impurity was identified as the mono-substituted product $Br(CD_2)_3I$).

2.3.2. 1,1′-(d_6-Propane-1,3-diyl)bis(4-tert-butylpyridinium) diiodide (d_6-MB327 I_2)

d_6-1,3-Diiodopropane (6.04 g, 20 mmol) was added dropwise to a stirred solution of 4-*tert*-butylpyridine (8.10 g, 60 mmol) in nitromethane (30 ml) in a 100-ml round-bottomed flask. The mixture was heated for 18.25 h. TLC analysis of a sample versus the starting materials, eluting with 1:2 hexane–acetone, was performed. None of the d_6-1,3-diiodopropane was seen upon developing the plates with iodine vapour; a main spot at retention factor (R_f) 0.05 (desired product), a fainter one at R_f 0.15

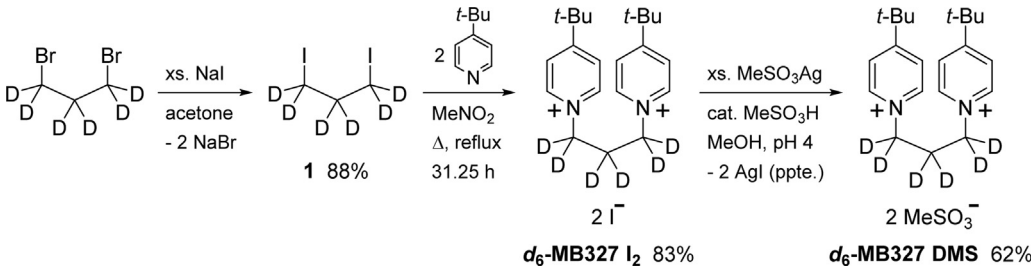

Fig. 2. Synthesis of the deuterated internal standard d6-MB327 DMS.

(mono-substitution product), and a spot corresponding to the excess of 4-*tert*-pyridine, were observed. The mixture was heated under reflux for a further 13 h, giving a total reflux time of 31.25 h. TLC of the mixture differed little from the previous analysis, signifying that the reaction was essentially complete. The solvent was removed from the mixture to yield a solid which was dried further using a vacuum pump. The solid obtained was dissolved in the minimum volume of hot isopropanol and left to cool slowly. The pale orange crystals produced were filtered off and dried to constant weight using the vacuum pump, and comprised the pure product (9.50 g, 83%). Mp 241–242 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 9.72–9.69 (4H, m, 2- and 6-H), 7.95–7.93 (4H, m, 3- and 5-H), 1.42 (18H, s, 6 × CH_3) ppm.

2.3.3. 1,1′-(d_6-Propane-1,3-diyl)bis(4-tert-butylpyridinium) dimethanesulfonate (d_6-MB327 DMS)

The previous compound (4.58 g, 8.0 mmol) and methanol (100 ml) and a magnetic stirrer bead were added to a 500-ml two-necked round-bottomed flask equipped with a dropping funnel and condenser topped by a guard tube (containing anhydrous calcium chloride). The stirred mixture was heated to an external temperature of 60 °C using an oil bath until full dissolution of the d_6-MB327 I_2 occurred. A few drops of methanesulfonic acid were added to the resulting solution to adjust the pH to 4. A solution of silver(I) methanesulfonate (3.31 g, 16.3 mmol) in acetonitrile (35 ml) was added dropwise with stirring at 60 °C over 25 min. A sticky and stringy precipitate formed on addition. By the end of the addition this had been replaced by a yellow granular precipitate. Stirring at 60 °C was continued for a further 90 min. The mixture was allowed to cool to room temperature and stirred at ambient temperature for 12 h. The precipitate of silver(I) iodide was filtered off and rinsed thoroughly with methanol. The solvent was removed from the filtrate and the crude product – a viscous pale-brown liquid – was dried using a vacuum pump. This crude product (4.54 g) was dissolved in the minimum volume of isopropanol (~8 ml), allowed to cool, anhydrous diethyl ether added until the mixture started to become turbid, and swirled while cooling in an acetone–cardice bath at −78 °C. Crystals formed slowly. The mixture was left overnight at ambient temperature. The white crystals obtained were filtered off, rinsed with anhydrous diethyl ether, and dried to constant weight at ~130 °C using a vacuum pump. Analytically pure product was isolated as a white solid (2.50 g, 62%). Mp 180–185 °C. ^{1}H NMR (400 MHz, $CDCl_3$) δ = 9.67 (4H, d, J = 6.9 Hz, 2- and 6-H), 7.86 (4H, d, J = 6.9 Hz, 3- and 5-H), 2.92 (6H, s, SCH_3), 1.36 (18H, s, 6 × CH_3) ppm.

2.4. Pharmacokinetics

2.4.1. Surgery

Under general anaesthesia (isoflurane) guinea-pigs were implanted with carotid cannulae (25 cm round-tip polyurethane, 2 Fr gauge (Instech-Solomon, USA)) which were exteriorised between the scapulae. The animals were connected using a harness and tether (Covance harness, Instech Solomon, USA) to a syringe and pump (CMA402, CMA microdialysis, Sweden) delivering heparinised saline (5 IU ml^{-1}, 0.5 μl min^{-1}) to maintain patency. The syringes and solution were changed daily. The animals were allowed to recover for 48 h prior to MB327 DMS administration.

2.4.2. Automated blood sampling

A total of 15 samples, each of 250 μl, were collected following administration of MB327 DMS (10 mg kg^{-1}, i.m.) using an automated blood sampling system (ABS; Instech Solomon, USA). Sampling time-points were: 5, 10, 15, 20, 30, 40, 50, 60, 75, 90, 120, 150, 180, 210 and 240 min. These were selected based on previous data obtained with the structurally similar compound HI-6 (Mumford et al., 2013).

2.4.3. Sample preparation

Following collection, blood samples were removed from the refrigerated ABS carousel and centrifuged for 1 min (13,300 revolutions min^{-1} ≡ 17,000 × g, in a Heraeus Pico 17 centrifuge, Thermo Fisher Scientific, UK); the plasma was then decanted from the red cell fraction into a separate vial. The samples were immediately frozen and then stored at −80 °C.

2.4.4. MB327 quantitation in plasma

Plasma samples (10 μl) were transferred to new Eppendorf tubes (Hamburg, Germany) and proteins precipitated by adding acetonitrile (200 μl containing 0.5 μg ml^{-1} of d_6-MB327 DMS). The contents of the tubes were vortex mixed for 30 s, then centrifuged for 5 min at 12,000 revolutions min^{-1} ≡ 10,000 × g. In each case, supernatant (150 μl) was transferred to a glass vial and evaporated to dryness under nitrogen using a TurboVap® LV automated evaporation system (Biotage; Uppsala, Sweden). Samples were reconstituted with 150 μl of acetonitrile–water (1:1 v/v containing 0.1% by volume of formic acid; Aldrich Ltd., Dorset, UK) and transferred to high recovery liquid chromatography (LC) vials for analysis using a Thermo Fisher Scientific Quantum TSQ® mass spectrometer (Thermo Fisher Scientific Oxoid Ltd., Hampshire, UK) equipped with a Thermo-Finnigan Surveyor HPLC pump and autosampler (Thermo-Finnigan; Hemel Hempstead, UK). An Obelisc R reverse-phase analytical LC column (SIELC Technologies; Wheeling, IL USA) of dimensions 150 mm × 2.1 mm × 5 μm was used and a guard column was fitted to the column inlet.

The mobile phases were A (40 mM ammonium formate, pH 3) and B (acetonitrile), and the chromatography eluent comprised 50% A and 50% B. The run time was 6 min and the flow rate was 0.5 ml min^{-1}. An injection volume of 5 μl was used. Concentrations of MB327 DMS ranging from 100 to 10,000 ng ml^{-1} in guinea-pig plasma (K2 EDTA, Harlan Laboratories UK Ltd., Oxfordshire) were used for calibration. The mass spectrometer was operated in electrospray ionisation (ESI) positive ion mode. A selected reaction monitoring method was created to monitor two transitions for the MB327 DMS analyte (m/z 156–105 and m/z 156–119) and

deuterated internal standard (m/z 159–108 and m/z 159–122). The scan time was 0.2 s per transition. The ESI voltage was set to 3500 V, sheath gas pressure to 50 l min^{-1}, auxiliary gas to 10 l min^{-1}, capillary temperature to 225 °C, capillary offset to 10, and the tube lens offset to 120 V.

For quantification, MB327 DMS peak area ratios were calculated using the internal standard and compared to those calculated from the calibration standards (Fig. 3). Data were processed using the Quant browser within the Thermo Xcalibur™ Software (Thermo Fisher Scientific Oxoid Ltd., Hampshire, UK).

2.5. Adverse effects

Some animals were dosed with MB327 DMS in the absence of nerve agent challenge to determine whether it produced adverse effects in unpoisoned animals. Following the initial assessment of adverse effects, MB327 (30 mg kg^{-1}) was administered in combination with atropine (3 mg kg^{-1}) and avizafone (3 mg kg^{-1}), in the absence of nerve agent. In these studies, signs of poisoning were observed closely for up to 2 h, with periodic checks to 4 h, at which point the surviving animals were culled by overdose of anaesthetic.

2.6. Efficacy study

The purpose of the experiment was to determine the 24-h LD_{50} of soman in the presence of MB327 I_2 or HI-6 DMS in combination with atropine and avizafone in the guinea-pig. The LD_{50} of soman in combination with atropine, avizafone and HI-6 was determined using the Dixon staircase method (Dixon, 1965). The LD_{50} values for atropine, avizafone and MB327 were derived using an adaptive probit method (Rice et al., 2015). Initially, the 24-h outcome determined the next challenge dose. Where an animal survived, the next dose in the curve was higher by one dose step, where an animal died the next dose in the curve was lower by one dose step. The dose step was fixed at 0.1 Log_{10} unit. Each determination was carried out in triplicate and an interim analysis was conducted at $n = 4$ per group. The remaining six animals in each determination were dosed at the predicted LD_{20}, LD_{50} and LD_{80} to provide a final dose-response relationship suitable for probit analysis.

2.6.1. Nerve agent and therapy administration

Soman was diluted in physiological saline (0.9% w/v) to the required concentration and administered subcutaneously between the scapulae at 1 ml kg^{-1}. Atropine (3 mg kg^{-1}) and avizafone (3 mg kg^{-1}) plus MB327 or HI-6 was administered 1 min following poisoning, intramuscularly in the thigh. The animals were observed periodically up to 6 h post-poisoning and again at 24 h post-poisoning, at which time surviving animals were culled by cervical dislocation.

A post-mortem examination of all soman-treated animals was carried out, with emphasis on the thoracic and abdominal organs. The examination looked for pulmonary haemorrhage and hyperinflation, and for intussusception in the gastrointestinal tract (Wetherell et al., 2007).

2.6.2. LD_{50} estimation and statistical analysis

This experiment used an adaptive model for generation of dose lethality data (Russell et al., 2009; Stallard, 2006). A probit regression model was used to fit a dose response curve to the survival data using the bias-reduced generalized linear model (brglm) method in the R statistical software package (R Core Team, 2014). The LD_{50} was calculated from the probit regression by rearranging the model formula in terms of dose (Russell et al., 2009; Stallard, 2006). Approximate standard errors for the fit to this dose estimate were calculated using the transformation method and confidence intervals using the delta method. At the conclusion of the study, all LD_{50} values were reanalysed to test for a dose-effect across the three treatment groups (Firth, 1993; Kosmidis, 2010). Comparisons of LD_{50} were made against the base of the model (atropine 3 mg kg^{-1}, avizafone 3 mg kg^{-1} and MB327 0 mg kg^{-1} group for MB327 and the LD_{50} in the absence of therapy for HI-6) to show differences between groups. A predetermined alpha level of 0.05 was taken to indicate a statistically significant difference in all cases.

3. Results

3.1. Pharmacokinetics

Following intramuscular administration of 10 mg kg^{-1} MB327 DMS reached C_{max} of 11 μg ml^{-1} (22 μM) at 12 min. The plasma

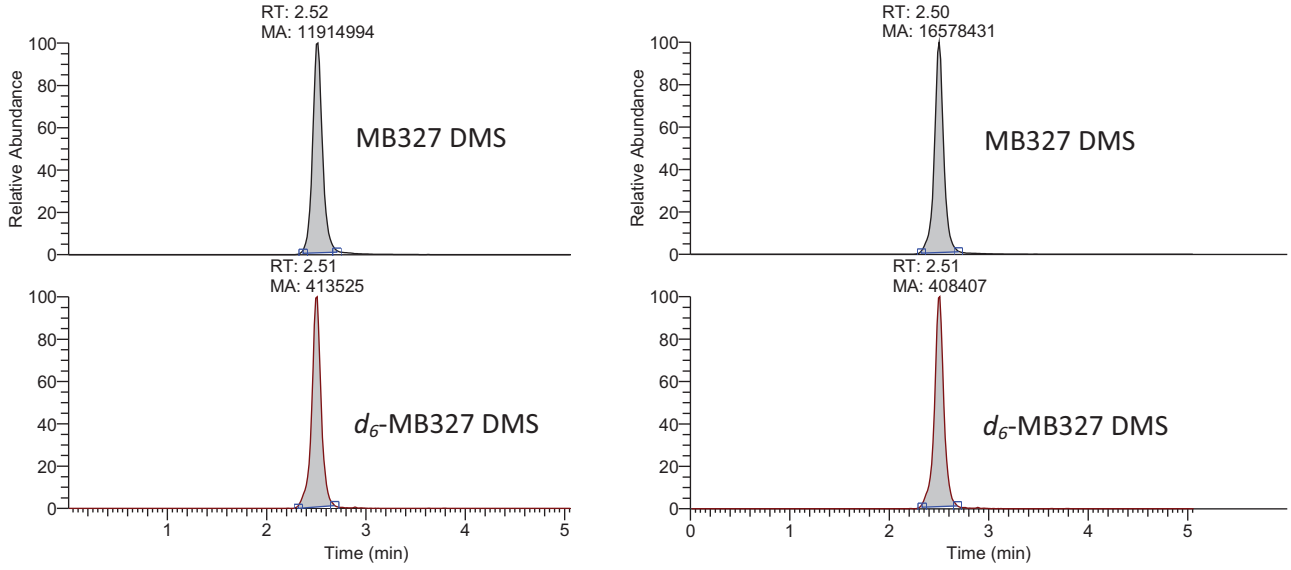

Fig. 3. Extracted ion chromatograms showing MB327 DMS in a sample of guinea-pig plasma taken for PK analysis (top left) versus guinea-pig plasma spiked with a known concentration of d6-MB327 DMS internal standard (bottom left), and the internal calibration standards for MB327 DMS (top right) and its deuterated analogue (bottom right) in control plasma. (RT = retention time, MA = mass area, of peak).

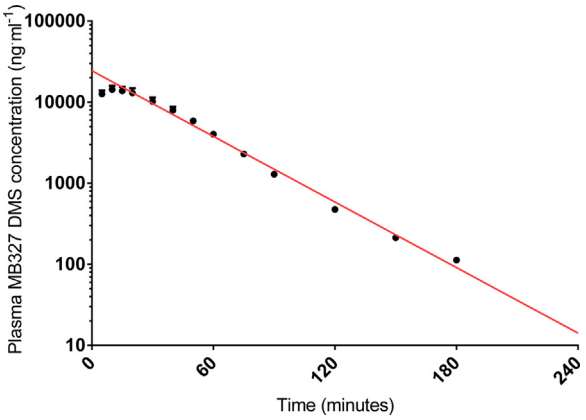

Fig. 4. Time-profile of concentration of MB327 DMS in guinea-pig plasma following its i.m. administration at 10 mg kg^{-1}. Data are shown as mean ± SEM (n = 7).

concentration profile is shown (Fig. 4). MB327 was eliminated with a half-life of 22 min.

3.2. Adverse effects

Any dose of MB327 DMS higher than 30 mg kg^{-1} caused acute respiratory failure leading to death within 30 min (Table 1). At 30 mg kg^{-1} respiration was impaired and mild flaccid paralysis evident. These animals recovered within 1 h. At a dose of 10 mg kg^{-1}, no adverse effects were observed. There was no difference in observed signs of poisoning in the group receiving atropine, avizafone and 30 mg kg^{-1} MB327 when compared to the group receiving 30 mg kg^{-1} MB327 alone.

3.3. Efficacy against soman poisoning

Both HI-6 and MB327 I_2 in combination with atropine and avizafone protected guinea pigs against the effects of soman. An increase in 24-h LD_{50} for subcutaneous soman with increasing MB327 or HI-6 dose was observed, this peaked at ~30 mg kg^{-1} for both compounds. A further increase in dose (to ~100 mg kg^{-1}) provided no further increase in protection. The increase in LD_{50} was statistically significant compared to the atropine/avizafone treatment group ($p < 0.01$) for MB327 at 33.9 mg kg^{-1}. In the HI-6-treated groups, the LD_{50} was significantly increased compared to the soman group 30 and 100 mg kg^{-1} doses (Fig. 5). Intussusception occurred in a few animals (n = 11), but there was no correlation with either agent or therapy dose (n = 3 at 0 mg kg^{-1}; n = 2 at 11.3 and 33.8 mg kg^{-1}; n = 4 at 113 mg kg^{-1}).

The distribution of times to death after lethal doses of soman, in each therapy dose group, probably reflects the adverse effects of MB327 (Table 2). The optimal therapy dose (33.8 mg kg^{-1}) prevented death for at least 6 h in all animals. However, at the higher dose (113 mg kg^{-1}) deaths occurred earlier, in the 0–1 h period. As this dose was toxic in the absence of nerve agent, the early deaths cannot necessarily be attributed to nerve agent poisoning.

Table 1
Survival time of guinea-pigs administered MB327 DMS. Guinea pigs were administered the indicated dose of MB327 alone or in combination with atropine sulfate (3 mg kg^{-1}) and avizafone (3 mg kg^{-1}) (30+). n.a. - surviving animals were all in good condition at the end of the observation period (4 h).

MB327 DMS dose (mg kg^{-1})	Mean time to death ± SD (min)	n
500	5.3 ± 0.7	4
250	12.6 ± 8.1	4
100	17.1 ± 4.0	4
30	n.a.	6
30+	n.a.	6
10	n.a.	6

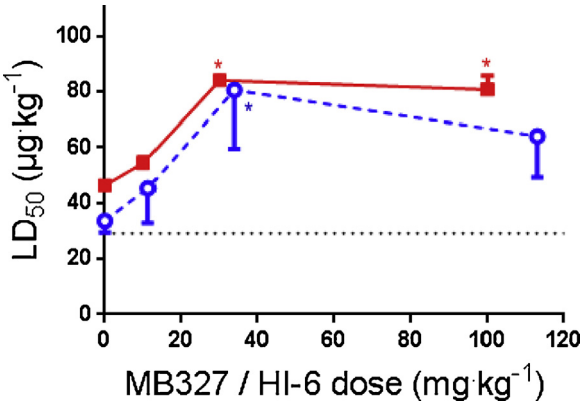

Fig. 5. 24-h LD_{50} for subcutaneous soman in the guinea-pig following treatment with atropine (3 mg kg^{-1}), avizafone (3 mg kg^{-1}) and the indicated dose of either MB327 I_2 (blue open circles and dashed line) or HI-6 DMS (red closed squares). *$p < 0.05$ vs. LD_{50} in the absence of therapy (HI-6) or, vs. atropine and avizafone therapy. The 0 mg kg^{-1} dose for each treatment indicates atropine + avizafone. Dashed line indicates the LD_{50} for soman in the absence of therapy. Data are shown as mean ± SE.

4. Discussion

When given intramuscularly in combination with atropine and avizafone, both MB327 I_2 and the oxime HI-6 DMS provided similar protection following acute (subcutaneous) soman poisoning. In both cases, the protection ratio (LD_{50} with treatment/LD_{50} without treatment) achieved by the optimal dose of each compound was approximately 2.8.

Although there is some non-competitive antinicotinic effect attributable to HI-6 (Tattersall, 1993), it is less potent and effective than MB327 in terms of providing direct functional recovery in soman-poisoned neuromuscular preparations in vitro (Seeger et al., 2011; Tattersall, 1993; Turner et al., 2011), and therefore, given its rapid administration following poisoning it is likely that the protective effect of HI-6 is attributable to reactivation of unaged AChE in the guinea pig. MB327 I_2 lacks an oxime group and thus its mechanism of action cannot involve reactivation of inhibited AChE (Schoene et al., 1976). Given that beneficial antinicotinic effects are exhibited by MB327 in vitro it follows that this action may be responsible for the protection seen in vivo. Such action is desirable because, by acting downstream of AChE inhibition, it should provide broad spectrum protection against any nerve agent (Timperley et al., 2012; Turner et al., 2011) and may still be effective following a delay in its administration, whereas ageing of some agent-AChE complexes would provide resistance to an oxime-based therapy following such a delay.

Male animals were used in this study to control for the potential effects of gender and hormonal changes on variability of response. A comparison of the toxicity of three nerve agents in male and female guinea pigs (Fawcett et al., 2009) demonstrated no difference in toxicity of soman in male and female guinea pigs, although there was variation in toxicity of sarin and VX with gender and age. Where efficacy studies of HI-6 directly comparing male and female animals have been carried out there were no differences in guinea pigs, although there were marked differences between male and female rats (Lundy et al., 1989).

Although not used in this study, pretreatment with pyridostigmine bromide is part of the UK provision of medical countermeasures to nerve agents (Timperley and Tattersall, 2015). As a combination of antimuscarinic (hyoscine), MB327 and carbamate

Table 2
Percentage of animals dying at <1 h, 1–6 h, 6–24 h or alive at 24 h following soman (at all doses used, dose range shown in final column) and therapy comprising atropine (3 mg kg^{-1}), avizafone (3 mg kg^{-1}) and the indicated dose of MB327 I_2.

MB327 (mg kg^{-1})	%Dead <1 h	%Dead 1–6 h	%Dead 6–24 h	%Alive 24 h	Agent dose range (μg kg^{-1})
0 (n = 15)	26.7	6.7	33.3	33.3	22.8–57.5
11.3 (n = 21)	23.8	4.8	33.3	38.1	25.8–182
33.8 (n = 18)	0	0	66.7	33.3	51.1–232
113 (n = 18)	38.9	11.1	27.8	22.2	45.7–232

(physostigmine) was effective as a therapy in guinea-pigs post-poisoning with soman (Timperley et al., 2012; Turner et al., 2011), there is no reason to believe that MB327 therapy would be incompatible with a carbamate pretreatment.

MB327 and related bispyridinium compounds have been shown to affect a number of other targets that could conceivably provide protection following nerve agent poisoning including mAChRs (Niessen et al., 2012b), nAChR orthosteric sites (Niessen et al., 2013) and AChE (Niessen et al., 2013, 2011). Antagonism at mAChRs classically provides the mainstay of protection against nerve agent poisoning and MB327 shows a similar or greater affinity for inhibition of mAChRs than for its channel block observed at nAChR (Königer et al., 2013; Mawdsley and Green, 2015; Niessen et al., 2012b). In previous studies and those reported here, relatively high doses of antimuscarinic (hyoscine or atropine) were used and it is unclear if MB327 would provide additional effective muscarinic inhibition over and above that provided by these antimuscarinics. It has recently been suggested that the atropine concentrations achieved clinically in patients being treated for OP poisoning may not fully block all muscarinic actions, at least in the isolated ileum preparation used, and that a degree of additional muscarinic block provided by MB327 could be achieved (Königer et al., 2013). Careful characterisation of both the PK and PD of both the antimuscarinic and MB327 in the animal model of nerve agent poisoning is likely to be required to clarify which mechanism is responsible for the protection. Additionally, the identification of compounds with actions similar to MB327 at nAChRs, but which exhibit better nicotinic selectivity, could also provide further clarification of this mechanism. MB327 has no effect on the inhibition of AChE by soman (Schoene et al., 1976) and, whilst the early administration of a high potency carbamate (physostigmine) AChE inhibitor has been shown to be beneficial following nerve agent poisoning (Wetherell et al., 2006, 2007), it is unlikely that MB327 administration post-exposure would have a protective effect given its very low potency at this target (IC_{50} ~600 μM) (Niessen et al., 2013).

A major finding in this study has been the observation of severe adverse effects at doses of MB327 very similar to those that provide protection. Animals administered MB327 DMS alone exhibited a flaccid paralysis and respiratory difficulties which were rapidly fatal at the highest doses tested. These signs of poisoning are consistent with an antinicotinic neuromuscular block possibly due to interactions at the ion channel, the orthosteric ACh binding sites or at both. There does appear to be some mutual antagonism between AChE inhibition by soman and the adverse effects of MB327 as demonstrated by the survival in soman-poisoned animals treated with the highest dose of MB327. In the absence of soman poisoning a very robust and rapid lethality of MB327 DMS was observed in all animals. A similar effect is seen with atropine. The atropine dose (2 mg) in the L4A1ComboPen® autoinjector issued to UK forces for self-/buddy-aid is deemed to be the maximum intramuscular dose compatible with military activities (Cullumbine et al., 1952). This is due to the adverse effects of atropine in the absence of nerve agent, where doses as low as 5 mg may cause a sensation of dizziness, giddiness or light-headedness (Cullumbine et al., 1955; McKee et al., 1952) and doses of 10 mg may cause delirium (Ostfeld et al., 1959, 1960). These doses are, however, lower than those which could be employed in a clinical setting, where doses of up to 200 mg intravenously over 10–15 min have been used to treat nerve agent intoxication (Foroutan, 1997; Newmark, 2004).

No attempt has been made in these studies to optimise aspects of the MB327 administration regimen other than dose. The pharmacokinetics for MB327 are presented here for the first time and are similar to those of related oximes (Mumford et al., 2013) with clearance from the circulation being rapid. This is not ideal for protection via an antinicotinic action, as the continued presence of the drug is required for it to exert its beneficial actions via channel block (Tattersall, 1993; Turner et al., 2011). In addition, the relatively high C_{max} resulting from the bolus administration may be responsible for the adverse effects via excessive nicotinic receptor antagonism.

The adverse effects observed in this study may make it difficult to use this particular compound as a therapy against nerve agent poisoning. However, careful infusion and titration to effect could be used to achieve a better pharmacokinetic profile more suited to the hypothesised mechanism of action whilst avoiding adverse effects. Such an approach may be better aimed at casualty management in medical care facilities, where sustained administration by (for example) intravenous or intraosseous infusion or a suitably formulated sustained release drug preparation could be used. Animal studies should be carried out in order to determine the efficacy of such an approach. Additionally, efforts to improve the understanding of the structural and chemical characteristics defining nicotinic channel interactions may lead to development of compounds with increased efficacy and improved safety profiles.

5. Conclusions

A non-competitive nicotinic ion channel blocker (MB327), in combination with an antimuscarinic (atropine) and an anticonvulsant (avizafone), protected guinea-pigs against soman toxicity with an efficacy equivalent to that of the oxime HI-6. It is hypothesised that the antinicotinic action of MB327 is responsible for the protection observed, although interactions at other targets involved in cholinergic signalling may contribute. MB327 was not without adverse effects, these were severe and probably resulted from block of neuromuscular transmission of the respiratory muscles. MB327 was rapidly cleared from the circulation potentially limiting its protective effect. These data suggest that PK and adverse effect profiles of any additional candidate compounds should be conducted alongside further efficacy studies in the assessment of these compounds as promising broad-spectrum nerve agent antidotes.

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References

Cullumbine, H., McKee, W.H.E., Creasey, N.H., 1955. The effects of atropine sulphate upon healthy male subjects. Quart. J. Exp. Physiol. Cogn. Med. Sci. 40, 309–319.
Cullumbine, H., McKee, W.H.E., Cruickshank, J.D., Lacon, H.W., Wybergh, J.G.M., 1952. The Effect of Atropine Sulphate on Military Efficiency. PTP 310, WO189/648. Chemical Defence Establishment, Porton, UK.
Dixon, W.J., 1965. The up-and-down method for small samples. J. Am. Stat. Assoc. 60, 967–978.
Fawcett, W.P., Aracava, Y., Adler, M., Pereira, E.F.R., Albuquerque, E.X., 2009. Acute toxicity of organophosphorus compounds in guinea pigs is sex- and age-dependent and cannot be solely accounted for by acetylcholinesterase inhibition. J. Pharmacol. Exp. Ther. 328, 516–524.
Firth, D., 1993. Bias reduction of maximum-likelihood-estimates. Biometrika 80, 27–38.
Fleisher, J.H., Harris, L.W., Clark, J.H., 1965. Dealkylation and aging of cholinesterase (ChE) following inhibition with pinacolyl methylphosphonofluoridate (Soman). Fed. Proc. 24, 641.
Foroutan, S., 1997. Medical notes on chemical warfare, part IV (in Farsi). Kowsar Med. J. 2, 141–151.
Heilbronn, E., Tolagen, B., 1965. Toxogonin in Sarin, Soman and Tabun poisoning. Biochem. Pharmacol. 14, 73–77.
Königer, C., Worek, F., Thiermann, H., Wille, T., 2013. Effect of MB327 and oximes on rat intestinal smooth muscle function. Chem. Biol. Interact. 204 (1), 1–5.
Kosmidis, I., 2010. brglm: bias reduction in binary-response GLMs. http://www.ucl.ac.uk/~ucakiko/software.html (accessed April, 2015.).
Lundy, P.M., Goulet, J.C., Hand, B.T., 1989. Hormone-dependent and dose schedule-dependent protection by HI-6 against soman and tabun poisoning. Fundam. Appl. Toxicol. 12, 595–603.
Mawdsley, D., Green, A.C., 2015. Affinity of the Bispyridinium Compound MB327 at the Muscarinic Acetylcholine Receptor. Unpublished Observations.
McKee, W.H.E., Cullumbine, H., Creasey, N.H., 1952. Effects of Atropine Sulphate upon Healthy Male Subjects. PTP 294, WO189/634. Chemical Defence Establishment, Porton Down, UK.
Mumford, H., Docx, C.J., Price, M.E., Green, A.C., Tattersall, J.E.H., Armstrong, S.J., 2013. Human plasma-derived BuChE as a stoichiometric bioscavenger for treatment of nerve agent poisoning. Chem. Biol. Interact. 203, 160–166.
Newmark, J., 2004. The birth of nerve agent warfare—lessons from Syed Abbas Foroutan. Neurology 62, 1590–1596.
Niessen, K.V., Seeger, T., Tattersall, J.E.H., Timperley, C.M., Bird, M., Green, C., Thiermann, H., Worek, F., 2013. Affinities of bispyridinium non-oxime compounds to [^{3}H] epibatidine binding sites of *Torpedo californica* nicotinic acetylcholine receptors depend on linker length. Chem. Biol. Interact. 206, 545–554.
Niessen, K.V., Seeger, T., Thiermann, H., Worek, F., 2012a. Interaction of bispyridinium compounds with the orthosteric binding site of human alpha 7 nicotinic acetylcholine receptors (h alpha 7 nAChRs). Naunyn. Schmiedebergs Arch. Pharmacol. 385, 63–64.
Niessen, K.V., Tattersall, J.E.H., Timperley, C.M., Bird, M., Green, C., Seeger, T., Thiermann, H., Worek, F., 2011. Interaction of bispyridinium compounds with the orthosteric binding site of human alpha 7 and *Torpedo californica* nicotinic acetylcholine receptors (nAChRs). Toxicol. Lett. 206, 100–104.
Niessen, K.V., Tattersall, J.E.H., Timperley, C.M., Bird, M., Green, C., Thiermann, H., Worek, F., 2012b. Competition radioligand binding assays for the investigation of bispyridinium compound affinities to the human muscarinic acetylcholine receptor subtype 5 (hM5). Drug Test. Anal. 4, 292–297.
Ostfeld, A., Jenkins, R., Pasnau, R., 1959. Dose–response data for autonomic and mental effects of atropine and hyoscine. Fed. Proc. 18 430–430.
Ostfeld, A.M., Machne, X., Unna, K.R., 1960. The effects of atropine on the electroencephalogram and behavior in man. J. Pharmacol. Exp. Ther. 128, 265–272.
R Core Team, 2014. R: A Language and Environment for Statistical Computing. Foundation for Statistical Computing, Vienna, Austria.
Rice, H., Dalton, C.H., Price, M.E., Graham, S.J., Green, A.C., Jenner, J., Groombridge, H. J., Timperley, C.M., 2015. Toxicity and medical countermeasures studies on the organophosphorus nerve agents VM and VX. Proc. R. Soc. A 471, 20140891.
Russell, K.G., Eccleston, J.A., Lewis, S.M., Woods, D.C., 2009. Design considerations for small experiments and simple logistic regression. J. Stat. Comput. Simul. 79, 81–91.
Schoene, K., Steinhanses, J., Oldiges, H., 1976. Protective activity of pyridinium salts against soman poisoning in vivo and in vitro. Biochem. Pharmacol. 25, 1955–1958.
Seeger, T., Eichhorn, M., Lindner, M., Niessen, K.V., Tattersall, J.E.H., Timperley, C.M., Bird, M., Green, A.C., Thiermann, H., Worek, F., 2012. Restoration of soman-blocked neuromuscular transmission in human and rat muscle by the bispyridinium non-oxime MB327 in vitro. Toxicology 294, 80–84.
Seeger, T., Niessen, K., Gerhardus, J., Langer, P., Gonder, S., Worek, F., Thiermann, H., 2011. Correlation of muscle strength and AChE activity in diaphragm of rat and guinea pig as a tool for therapy development in nerve agent poisoning. Naunyn. Schmiedebergs Arch. Pharmacol. 383 14–14.
Stallard, N., 2006. Optimal adaptive designs for acute oral toxicity assessment. J. Stat. Plan. Infer. 136, 1781–1799.
Tattersall, J.E.H., 1993. Ion channel blockade by oximes and recovery of diaphragm muscle from soman poisoning in vitro. Br. J. Pharmacol. 108, 1006–1015.
Timperley, C.M., Banks, R.E., Young, I.M., Haszeldine, R.N., 2011. Synthesis of some fluorine-containing pyridinealdoximes of potential use for the treatment of organophosphorus nerve-agent poisoning. J. Fluor. Chem. 132, 541–547.
Timperley, C.M., Bird, M., Green, A.C., Price, M.E., Chad, J.E., Turner, S.R., Tattersall, J.E. H., 2012. 1,1′-(Propane-1,3-diyl)bis(4-*tert*-butylpyridinium)di (methanesulfonate) protects guinea pigs from soman poisoning when used as part of a combined therapy. Med. Chem. Commun. 3, 352–356.
Timperley, C.M., Bird, M., Heard, S.C., Notman, S., Read, R.W., Tattersall, J.E.H., Turner, S.R., 2005. Fluorinated pyridine derivatives. Part 1: the synthesis of some mono- and bis-quaternary pyridine salts of potential use in the treatment of nerve agent poisoning. J. Fluor. Chem. 126, 1160–1165.
Timperley, C.M., Tattersall, J.E.H., 2015. Toxicology and medical treatment of organophosphorus compounds. Best Synthetic Methods: Organophosphorus (V) Chemistry. Elsevier, Oxford, UK.
Turner, S.R., Chad, J.E., Price, M., Timperley, C.M., Bird, M., Green, A.C., Tattersall, J.E. H., 2011. Protection against nerve agent poisoning by a noncompetitive nicotinic antagonist. Toxicol. Lett. 206, 105–111.
Wetherell, J., Price, M., Mumford, H., 2006. A novel approach for medical countermeasures to nerve agent poisoning in the guinea-pig. Neurotoxicology 27, 485–491.
Wetherell, J., Price, M., Mumford, H., Armstrong, S., Scott, L., 2007. Development of next generation medical countermeasures to nerve agent poisoning. Toxicology 233, 120–127.