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Protection against nerve agent poisoning by a noncompetitive nicotinic antagonist

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ABSTRACT

The acute toxicity of organophosphorus (OP) nerve agents arises from accumulation of acetylcholine (ACh) and overstimulation of ACh receptors. The mainstay of current pharmacotherapy is the competitive muscarinic antagonist, atropine. Nicotinic antagonists have not been used due to the difficulties of administering a dose of a competitive neuromuscular blocker sufficient to antagonise the effects of excessive ACh, but not so much that it paralyses the muscles. An alternative approach would be to use a noncompetitive antagonist whose effects would not be overcome by increasing ACh concentrations. This study demonstrates that the compound 1,1′-(propane-1,3-diyl)bis(4-*tert*-butylpyridinium), which blocks open nicotinic ion channels noncompetitively, is able to reverse the neuromuscular paralysis after nerve agent poisoning *in vitro* and to protect guinea pigs against poisoning by nerve agents when used as part of a therapeutic drug combination including a muscarinic antagonist. In contrast to the oxime HI-6, this compound was equally effective in protecting against poisoning by sarin or tabun. Further studies should identify more effective compounds with this action and optimise doses for protection against nerve agent poisoning *in vivo*.

1. Introduction

The acute toxicity of organophosphorus (OP) nerve agents arises from accumulation of acetylcholine (ACh) at both muscarinic and nicotinic ACh receptors (mAChRs and nAChRs) due to inhibition of acetylcholine esterase (AChE); however, current pharmacotherapy focuses only on the mAChR component, the mainstay of treatment being the competitive muscarinic antagonist, atropine. The nicotinic effects are treated indirectly, by the use of an oxime to reactivate inhibited AChE. The search for the ideal oxime has been hampered by the fact that no single oxime demonstrates adequate reactivating activity against all the known nerve agents. Whichever oxime is used will always be deficient against one or more of the nerve agents, making a generic therapy unlikely (Bajgar et al., 2007).

The effectiveness of an appropriate antinicotinic drug to directly treat the nicotinic effects of poisoning should be independent of the nerve agent involved, in the same way as that of antimuscarinic drugs. The question of why nicotinic antagonists are not used (Smythies and Golomb, 2004) has been addressed by Sheridan et al. (2005), who emphasised the difficulties of administering a dose of competitive nicotinic neuromuscular blocker sufficient to antagonise the effects of excessive ACh, but not so much that it paralyses the muscles.

An alternative approach would be to use a noncompetitive antagonist whose effects would not be overcome by increasing concentrations of ACh. Certain bispyridinium compounds, including some oximes, have a beneficial effect in OP poisoning through this type of action, which correlates with their ability to block the open ion channel of the nAChR (Tattersall, 1993). Open channel block, a form of noncompetitive antagonism, is attractive because the block is use-dependent: antagonism becomes greater as channel activation increases. This is the converse of what happens with a competitive antagonist and appears to be an ideal way of mitigating the effects of overstimulation of nAChRs.

In this study, we demonstrate that a bispyridinium compound blocks open nicotinic ion channels and that this noncompetitive antagonism, as well as reversing the neuromuscular blocking action of nerve agent *in vitro*, can protect animals against poisoning by nerve agents when used as part of a therapeutic drug combination.

2. Materials and methods

2.1. Drugs and chemicals

The iodide (MB327) and di(methanesulphonate) (MB399) salts of 1,1′-(propane-1,3-diyl)bis(4-*tert*-butylpyridinium) (Fig. 1) were synthesised at Dstl

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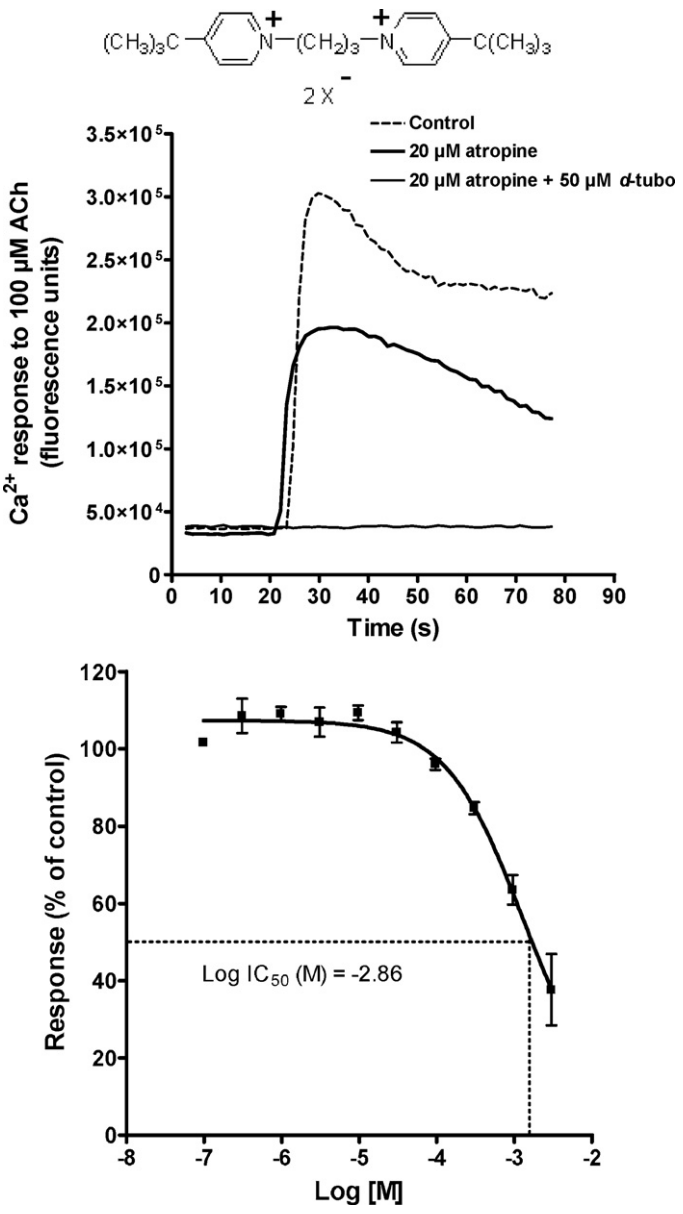

Fig. 1. Top: molecular formula of MB327/MB99 X = I or CH_3SO_3, respectively. Middle: representative calcium responses to acetylcholine (100 μM) in CN21 cells in the absence and presence of atropine (20 μM) and d-tubocurarine (50 μM). Bottom: effect of MB327 on the Ca^{2+} response to ACh, measured in CN21 cells using a FlexStation plate reader. Data are displayed as mean ± SEM of 4 experiments performed in duplicate on each plate ($n = 4$).

Porton Down and were >98% pure. For the sake of expediency, the diiodide salt was first prepared. These salts are easily accessible through quaternisation of two molar equivalents of the pyridine with 1,3-diiodopropane (Timperley et al., 2005) and many could be made in a short space of time for *in vitro* screening. As with bispyridinium oximes, the diiodide salts are not optimally soluble in water, unlike the corresponding dichloride or di(methanesulfonate) salts. For the *in vivo* experiments, where water solubility is important for dose formulation, the diiodide salt (MB327) was converted into the more soluble di(methanesulfonate) salt (MB399). The nature of the counterion is expected to have a negligible effect on the ion channel blocking activity as the latter depends on the nature of the cation.

The nerve agents sarin, soman and tabun (>95% purity) were also synthesised at Dstl Porton Down. HI-6 di(methanesulphonate) was supplied by Edinburgh Pharmaceutical Processes (Edinburgh, UK). All proprietary drugs and chemicals were purchased from Sigma–Aldrich Chemical Company UK (Poole, Dorset, UK).

2.2. Cell culture

CN21 cells, derived from the TE671 human rhabdomyosarcoma cell line by a stable transfection of the ε-subunit to express both the foetal and adult human-muscle nicotinic receptor, were obtained from Dr. David Beeson (Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, UK) (Beeson et al., 1996). The cells were grown using standard cell culture techniques in Dulbecco's modified Eagle's Medium (Sigma–Aldrich, UK) with 10% Foetal Bovine Serum (Invitrogen, UK), 50 units/ml penicillin, 50 μg/ml streptomycin, 2 mM L-glutamine (Sigma–Aldrich, UK) and 0.5 mg/ml geneticin (Invitrogen, UK) and grown in 150 cm^2 cell culture flasks until approximately 70–80% confluent in a humidified atmosphere in an incubator at 36.5 °C with 5% CO_2. Cells were then harvested using 0.25% trypsin/ethylenediamine tetraacetic acid (EDTA) solution (Sigma–Aldrich, UK) in Ca^{2+}/Mg^{2+}-free phosphate buffered saline and were collected by centrifugation ($80 \times g_{max}$, 4 min). For maintenance, cells were re-plated into culture flasks at split ratios of 1:6–1:10. Cells were used in experiments between passages 2 and 8 following recovery from cryopreservation.

2.3. Nicotinic calcium response assay

The methods used were similar to those developed by Ring and Strom for experiments with nicotinic receptor inhibitors in SH-SY5Y cells (personal communication, Ring A and Strom BO, manuscript in preparation). CN21 cells were plated out onto clear-bottomed, black-walled, tissue culture treated 96-well plates (Corning® Costar®) at a density of ~20,000 cells per well in 100 μl of medium (so that they approached confluence after 24 h) for subsequent experimentation. The growth medium was removed, leaving the cells adhering to the bottom of the plate. 50 μl of a Calcium 4 assay kit dye (Fluo-4 acetoxymethylester, Molecular Devices FLIPR Calcium 4 assay kit, Molecular Devices, Union City, CA, USA) in a HEPES-buffered balanced salt solution (NaCl 135 mM, KCl 5.4 mM, $CaCl_2$ 1 mM, $MgCl_2$ 1 mM, HEPES acid 5 mM, $NaHCO_3$ 3.6 mM, and D-glucose 10 mM, pH 7.4 with NaOH) was added and the cells were incubated in the dark for ~30 min. Cells were not washed prior to assay as the FLIPR Calcium 4 assay kit also contains a quenching dye to minimise fluorescence from extracellular de-esterified (fluorescent) Fluo-4. The dye loading solution also contained atropine (20 μM) to block the muscarinic response and pharmacologically isolate the nicotinic response to ACh.

MB327 was dissolved as a stock solution in HEPES-buffered solution on the day of the experiment. After the cells had been incubated with the dye, dilutions of this stock solution were added to each well on the cell plate to achieve the final desired concentrations. The first and last columns received a vehicle control so that the compound response could be compared to the vehicle at the beginning and end of each experiment to allow evaluation of time-dependent effects. MB327 was tested at 10 different concentrations in duplicate on each plate and each experiment was repeated 3 times ($n = 3$).

For testing, plates were transferred to a FlexStation II fluorescence plate reader (Molecular Devices) and the fluorescence intensity was measured at ~1 s intervals prior to and after addition of 58 μl of 20 μM ACh to achieve a final concentration in each well of ~10 μM, which gave approximately 80% of the maximal response (EC_{80}). Excitation and emission wavelengths were set to 485 nm and 525 nm respectively, with a cut-off of 515 nm. All measurements were made at room temperature (~21 °C). Agonist additions were made automatically using the plate reader's inbuilt dispensing functions. Baseline fluorescence was measured for 20 s, followed by addition of ACh. Further measurements of fluorescence for 80 s were then made to follow the agonist-induced response.

Responses were quantified as the maximum response expressed as a percentage of the average baseline values. For analysis, these data were then normalised to the mean vehicle control values for the wells in the corresponding row. Responses were fitted to a standard four parameter logistic equation using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA) and a log IC_{50} and Hill slope with standard errors (SEM) were determined.

2.4. Single channel recordings

CN21 cells were plated out onto tissue culture treated 35 mm Petri dishes prior to experimentation. For recording, the culture medium was replaced with an extracellular solution composed of (mM): NaCl 135, KCl 5.4, $CaCl_2$ 1, $MgCl_2$ 1, HEPES acid 5 (titrated to pH 7.2 with NaOH). Patch pipettes (5–10 MΩ) were pulled from borosilicate glass and coated with Sylgard® to improve the signal-to-noise ratio. After fire-polishing, pipettes were filled with a solution comprising (mM): KCl 140, $CaCl_2$ 1, $MgCl_2$ 1, ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) 11, HEPES acid 5 (titrated to pH 7.2 with KOH).

Single channel currents were recorded from isolated 'outside-out' patches with an Axopatch 200B amplifier (Molecular Devices). Signals were filtered using a 4-pole Bessel (−3 dB cut-off 5 kHz), digitised at 25 kHz by a Digidata 1200 and recorded using pClamp 9 (both from Molecular Devices). All experiments were performed at room temperature (~21 °C), as this meant the reaction kinetics were slower, making channel opening and closing events easier to follow with the recording system. Each patch was continuously perfused at a flow rate of approximately 0.1–0.2 ml/min with the extracellular solution, followed by one containing 0.2 μM ACh to ensure