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O-Substituted derivatives of pralidoxime: muscarinic properties and protection against soman effects in rats

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Abstract

O-Substituted aldoximes of the cholinesterase reactivator pralidoxime (*O*-methyl **1**, *O*-benzyl **2**, *O*-propynyl **3** and *O*-butynyl **4** derivatives) were synthesized and found to exhibit strong binding affinities for muscarinic receptors in rat brain, heart and submandibulary glands. The aldoximes were noncompetitive antagonists of acetylcholine-induced contraction of the guinea pig ileum. A good correlation was observed between binding affinity and pK_B. Weak anticholinesterase activities were observed for these compounds. When given intracerebroventricularly into conscious rats before soman administration (0.9 LD_{50}, subcutaneously), the aldoximes, like atropine but not pralidoxime, protected against respiratory depression (**3**,**4**) and bradycardia (**2**). No protection against soman-induced pressor effects was noted. The protective effects of these aldoximes may be the outcome of compensatory mechanisms, of which the cholinergic receptor agonist and antagonist properties of these compounds may be important.

Keywords: Pralidoxime derivative; Protection; Soman; Cholinergic property

1. Introduction

Anticholinesterase organophosphates are widely used as insecticides (parathion, malathion) and chemical warfare agents (sarin, soman, tabun). Organophosphate poisoning can occur in a variety of situations and can be accidental, suicidal or intentional. The immediate manifestation of organophosphate poisoning is the cholinergic syndrome due to inhibition of acetylcholinesterase. An intermediate syndrome may follow, of which the organophosphorus-induced delayed polyneuropathy is of particular importance (Taylor, 2001). Oxime reactivators like pralidoxime and 1-(2-hydroxyiminoethylpyridinium)-1-(4-carboxy-aminopyridinium)dimethyl ether (HI-6) are known to demonstrate protective effects against organophosphate poisoning that are unrelated to reactivation of the inhibited enzyme. HI-6 causes recovery of neuronal transmission in the respiratory centres of the brain and neurotransmission in the diaphragm of primates that have received lethal doses of soman or tabun (Van Helden et al., 1996). Pralidoxime given at concentrations that did not increase plasma cholinesterase activity reduced the incidence of necrotic fibres in rats treated with organophosphates metamidophos and isofenphos (Cavaliere et al., 1998).

The signs and symptoms of organophosphate intoxication are primarily due to the accumulation of acetylcholine in the synaptic cleft and the actions of the latter on acetylcholine receptors (Taylor, 2001). There are isolated reports that organophosphates like parathion and paraoxon bind to muscarinic receptors (Van den Beukel et al., 1997) and act as agonists at these sites (Van den Beukel et al., 1996; Ward and Mundy, 1996). Muscarinic receptor antagonists like atropine are particularly useful in counteracting the symptoms of organophosphate poisoning. There is evidence that the "direct" protective effects of aldoxime cholinesterase reactivators may be due to their ability to protect the post-synaptic cell from overstimulation by acetylcholine, possibly by blocking nicotinic ion channels or allosteric sites of central or peripheral muscarinic receptors (Van Helden et al., 1996). Pralidoxine has been reported to inhibit [^{3}H] quinuclidinyl benzilate binding and sodium-dependent high-affinity choline uptake in rat brain, although these effects were insufficient to ensure the survival of soman-treated rats (Shih et al., 1991). In this study, several

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O-substituted derivatives of pralidoxime have been synthesized. The objective is to investigate if these pralidoxime derivatives, which have greater antimuscarinic activity, would confer greater protective effects than pralidoxime in soman-treated mice.

2. Materials and methods

2.1. Chemistry

The synthesis and characterisation of the pralidoxime derivatives (O-methylpyridine-2-carbaldoxime methiodide **1**; O-benzylpyridine-2-carbaldoxime methiodide **2**; O-prop-2-ynylpyridine-2-carbaldoxime methiodide **3**; O-but-2-ynylpyridine-2-carbaldoxime methiodide **4**) have been reported earlier (Loke et al., 2001). The following parameters were determined from the energy minimized conformations of the aldoximes **1**–**4** and pralidoxime, obtained by minimization using the force field (MAXIMIN2) in SYBYL 6.2 (Tripos Associates, St. Louis, MO), with calculations continued until the root mean square (RMS) gradient was less than 0.001 kcal mol^{-1}: molecular volume, Connolly surface area, total dipole moment and negative charge on the oxime oxygen using the Gasteiger Huckel method, *C*log*P* (for the corresponding nonquaternised derivatives of aldoximes **1**–**4** and pralidoxime).

2.2. Muscarinic receptor binding studies

[^{3}H] Pirenzepine (NET-780, 78.8 Ci/mmol), [^{3}H] *N*-methylscopolamine chloride (NET-636, 82.0 Ci/mmol) and [^{3}H] oxotremorine-M acetate (NET-671, 85.8 Ci/mmol) were purchased from NEN Life Science Products (Boston, MA). Carbamylcholine chloride (carbachol), 2-pyridinealdoxime methochloride (pralidoxime), atropine sulfate, acetylthiocholine, dithiobisnitrobenzoic acid and acetylcholinesterase (electric eel, EC 3.1.1.7) were obtained from Sigma (St. Louis, MO). Tissue solubilizer (BTS-450) and scintillation cocktail (Ready-Organic) from Beckman were used as supplied. Male Sprague–Dawley rats, 250–300 g, were obtained from National University of Singapore, Laboratory Animals Centre.

Tissue homogenates from rat cerebral cortex, heart and submandibulary glands were prepared as previously reported (Xu et al., 1998). In a typical assay, an aliquot of the tissue homogenate was incubated with a fixed concentration of the radioligand (1 nM for [^{3}H] pirenzepine and [^{3}H] oxotremorine M, and 0.2 nM for [^{3}H] *N*-methylscopolamine) and different concentrations of the test compound (3.2 nM –8 mM) in a final volume of 1 ml Krebs–HEPES (20 mM) buffer (pH 7.4). In assays using [^{3}H] oxotremorine M as radioligand, HEPES buffer (20 mM, pH 7.4) was used. In the case of rat brain homogenates, 100 μl (equivalent to 2 mg protein/ml) was used. For the rat heart and submandibulary glands, 80 μl (0.5 mg protein/ml) and 40 μl (2.44 mg protein/ml) aliquots were used, respectively. The incubation (1 h, 22 °C) was terminated by centrifugation at 40 000 × g, 5 min, 4 °C. The supernatant was removed by gentle aspiration and the pellet washed once with ice-cold saline before the addition of tissue solubilizer (200 μl). The mixture was allowed to stand overnight, after which scintillation fluid was added and radioactivity determined on a Beckman LS 6100 Scintillation Counter. Nonspecific binding was determined in saturation studies using varying concentrations of radioligand ([^{3}H] pirenzepine 0.1–100 nM, [^{3}H] oxotremorine M 0.1–5.0 nM, [^{3}H] *N*-methylscopolamine 0.1–2.5 nM) in the presence of atropine (1μM), with other conditions remaining the same.

The displacement data were analysed using Graph Pad Prism™ 2.0 to derive the IC_{50} values for the aldoximes. The inhibitory constants K_i were calculated from IC_{50} values using the Cheng–Prusoff equation (Cheng and Prusoff, 1973). The saturation data were similarly analysed to give B_{max} and K_d values of the radioligands.

2.3. Functional in vitro studies on the guinea pig ileum

Female guinea pigs (280–350 g) were obtained from the National University of Singapore Laboratory Animals Centre. The animals were killed by cervical dislocation and the terminal portion (5 cm) of the ileum was removed and rinsed with Tyrode solution (composition in mM: NaCl 137, KCl 2.68, $CaCl_2$ 1.36, $MgCl_2$ 0.49, NaH_2PO_4 0.287, $NaHCO_3$ 11.9, glucose 5.55, aerated with CO_2 to give pH 7.2–7.6). A segment of 1–1.5 cm length was removed and suspended with a resting tension of 0.5 g in a 10-ml organ bath filled with Tyrode solution, maintained at 37 °C and aerated with 95% O_2–5% CO_2. After an equilibration period of 1 h, cumulative concentrations of acetylcholine (10 nM–200 μM) were added to obtain a concentration–dose response curve. This was repeated again on the same tissue. The acetylcholine-induced contractions were recorded with an isotonic transducer (Ugo Basile), attached to a MacLab 8 Virtual Instrument System via an amplifier. The test compound was then added to the bath for 10 min, after which the acetylcholine dose response curve was repeated in its presence. Each compound was tested in triplicates over the concentration range of 1 nM–1 mM. The dose response curves obtained in the presence/absence of the test compound were analysed using Graph Pad Prism™ 2.0 to obtain the inhibitory constant K_B for each compound.

2.4. Determination of anti-acetylcholinesterase activity

Anticholinesterase activity was determined by the method of Ellman et al. (1961) with some modifications. The inhibitor (10 μl, concentrations ranging from 10^{-1} to 10^{-7} M) was incubated with a solution of acetylcholinesterase (0.1 IU) in phosphate buffer (990 μl, 0.1 M, pH 7.4) for 3 min at 25 °C. After this time, an aliquot (100 μl) was removed and added to

phosphate buffer (0.1 M, pH 7.4) containing acetylthiocholine iodide (1 mM) and dithiobisnitrobenzoic acid (0.1 mM) in a total volume of 2 ml. The presence of enzymic activity will result in the hydrolysis of the substrate (acetylthiocholine) to thiocholine which reacts with dithiobisnitrobenzoic acid to form a yellow complex whose formation is monitored at 412 nM. Six different concentrations of each inhibitor were investigated in this way and the percentage inhibition was plotted against the logarithm of inhibitor concentration to give a sigmoidal curve from which IC_{50} could be determined.

2.5. Functional in vivo studies on cardiovascular and respiratory parameters of soman-treated rats

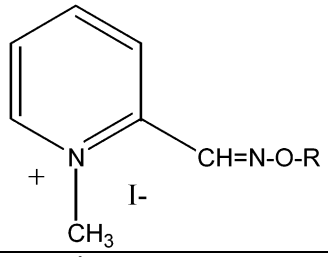
Male Sprague–Dawley rats, 250–300 g, obtained from the National University of Singapore Laboratory Animal Centre, were used for the experiments. The animals were handled according to international guidelines for animal research (Howard-Jones, 1985). They were adequately housed at temperatures of 22 ± 2 °C, humidity $55 \pm 5\%$ and a 12:12 h light/dark cycle with lights on at 7.00 a.m. Food and water were provided ad libitum.

A cannula was stereotaxically inserted into the brain (right ventricle, 1 mm posterior, 1.5 mm right lateral from bregma to a depth of 4 mm) of the rat to allow intracerebroventricular (i.c.v.) administration of the test compounds (Paxinos and Watson, 1997). Two to 4 days after the insertion, a telemetry transmitter device (Data Science, TA11-PA-C40) was implanted into the abdominal aorta of each rat under halothane anaesthesia, initially induced using a neurolepanalgesic cocktail of Hypnorm (Janssen, UK) and Dormicum (Roche, Switzerland). In this way, the test compounds can be administered directly into the brain of the rat and the cardiovascular and respiratory parameters can be collected simultaneously in the conscious animal. Since data collection of cardiovascular and respiratory parameters are made through remote telemetry means, it is possible to collect uninterrupted stress free data from the animal up to 4 h after soman administration.

The animal was acclimatized in the activity bowl for a duration of 1 h. Following this, the inner dummy probe on the guide cannula was replaced with a stainless steel injection cannula whose tip projected (3 mm) beyond the end of the guide cannula. During this process, the animal was not anaesthetized but held gently with one hand while the exchange was effected by the other hand. The injection cannula was connected to a 25-μl Hamilton syringe via a low dead volume tubing. Following an equilibration period of 30 min, the animal was administered with a bolus i.c.v. dose of 100 pmol angiotensin II in 10 μl saline to check for the correct placement of the injection cannula. Administration of angiotensin II into the lateral ventricles induced dipsosis and an increase in blood pressure lasting for about 6 min.

Twenty-four hours after the blood pressure has returned to basal value, saline (10 μl) was administered intracerebroventricularly to confirm the absence of Cushingnoid symptoms at the dosing rate used. The solvent vehicle (10% v/v dimethylsulfoxide in 0.9% w/v saline) was administered intracerebroventricularly to the rats 2 h before the administration of the test or control drug (atropine, carbachol). Blood pressure, heart rate and respiratory rate of the animal were monitored for 30 min following drug administration. This was followed by an injection (subcutaneous, s.c.) of soman at a dose equivalent to 0.9 LD_{50} ($LD_{50} = 110$ μg/kg) to each animal and the same parameters were monitored for a period of 4 h post-soman administration. Each compound was evaluated at three different dose levels (0.09, 1.67 and 4.5 μmol/kg), with three animals used for

Table 1
Physicochemical parameters of pralidoxime and derivatives **1**–**4**



Compound (R)	Connolly surface area (Å^2)[a]	Volume (Å^3)[a]	Total dipole moment[a]	Electron density on oxime oxygen[a]	Lipophilicity contribution of R substituent[b]
1 ($-CH_3$)	179.48	157.20	3.97	−0.209	0.13
2 ($-CH_2-C_6H_5$)	256.10	235.24	4.39	−0.190	1.90
3 ($-CH_2C \equiv CH$)	205.95	182.48	3.87	−0.193	0.23
4 ($-CH_2C \equiv C-CH_3$)	227.06	199.68	3.66	−0.193	0.76
Pralidoxime ($-H$)	158.94	138.23	3.92	−0.221	–

[a] Determined from energy minimized conformations of compound using MAXIMIN2 force field of SYBYL 6.2.

[b] Obtained from $ClogP_{\text{nonquaternised aldoxime}} - ClogP_{\text{nonquaternised PAM}}$. Energy minimized conformations of nonquaternised derivatives were similarly determined using the MAXIMIN2 force field of SYBYL 6.2. *C*log*P* of nonquaternised pralidoxime = 0.90.

Table 2
IC_{50} for acetylcholinesterase (AChE) inhibition, dissociation constants (pK_B) and binding affinities (K_i) of pralidoxime and related compounds (**1**–**4**)

Compound	Anti-AChE activity (IC_{50}, mM)[a]	pK_B[b]	K_i (μM) for inhibition of				PZ/Oxo-M ratio[d]
			[^{3}H] PZ binding in cortex[c]	[^{3}H] Oxo-M binding in cortex[c]	[^{3}H] NMS binding in heart[c]	[^{3}H] NMS binding in submandibulary glands[c]	
1	8.10 (2.70)	3.72	17.0	3.47	9.33	12.3	4.89
2	0.77 (0.40)	6.78	0.33	1.20	1.05	0.68	0.28
3	2.77 (0.98)	5.82	1.17	1.62	0.69	1.78	0.72
4	2.03 (0.21)	8.05	0.35	4.90	0.041	0.053	0.071
Pralidoxime	3.93 (0.50)	3.02	23.4	24.5	32.4	40.7	0.95
Carbachol			9.12	8.13×10^{-3}	8.13×10^{-3}	8.32	1122
Atropine		8.51[e]	5.01×10^{-4}	3.55×10^{-4}	3.55×10^{-4}	2.40×10^{-3}	1.41

[a] Values in parentheses represent S.D. for $n = 3$ determinations.
[b] $-\log K_B$ where K_B is the noncompetitive dissociation constant, determined from in vitro functional assays on guinea pig ileum.
[c] PZ = pirenzepine. Oxo-M = oxotremorine M. NMS = *N*-methylscopolamine. The K_d (dissociation constant) and B_{max} (maximal number of binding sites) for the following radiolabelled ligands on the specified tissue are: [^{3}H] pirenzepine (rat cortex, M_1) 15.83 nM, 1600 fmol/mg protein; [^{3}H] oxotremorine (rat cortex, M_1) 1.20 nM, 520 fmol/mg protein; [^{3}H] *N*-methylscopolamine (rat heart M_1) 0.30 nM, 1856 fmol/mg protein; [^{3}H] *N*-methylscopolamine (rat submandibulary glands, M_1/M_3) 0.10 nM, 563 fmol/mg protein. K_i values were determined from the average of three separate experiments.
[d] Ratios are calculated from K_i for the displacement of [^{3}H] pirenzepine (PZ, rat cortex) divided by K_i for displacement of [^{3}H] oxotremorine (Oxo-M, rat cortex).
[e] pA_2 (competitive dissociation constant).

each dose. The solvent vehicle had little influence on the parameters.

3. Results

The physicochemical parameters of pralidoxime and its *O*-substituted derivatives **1**–**4** are given in Table 1. *O*-Substitution of the aldoxime group predictably caused an increase in size of the molecule (Table 1). Little variation is noted in the dipole moments of the aldoximes as they are uniformly polar and quaternised molecules. Electron density on the oxime oxygen is decreased on *O*-substitution with marked decreases observed in the alkynyl derivatives **3** and **4** (possibly due to the electron withdrawing effect of the alkynyl triple bond) and the benzyl derivative **2**.

The binding affinities of aldoximes **1**–**4** were assessed from their ability to displace tritiated ligands from rat tissues which have a predominant population of a specific muscarinic subtype: [^{3}H] pirenzepine and [^{3}H] oxotremorine M from cerebral cortex (M_1); [^{3}H] ***N***-methylscopolamine from heart (M_2) and submandibulary glands (mix of M_1 and M_3). Receptor binding affinities are expressed in terms of the inhibitory constant K_i (Table 2). In the rat cortical tissue, [^{3}H] pirenzepine and [^{3}H] oxotremorine M have been used to label muscarinic receptor antagonist and agonist sites of the M_1 receptor, respectively (Ward et al., 1995). The ratio of K_i values for the displacement of [^{3}H] pirenzepine to [^{3}H]

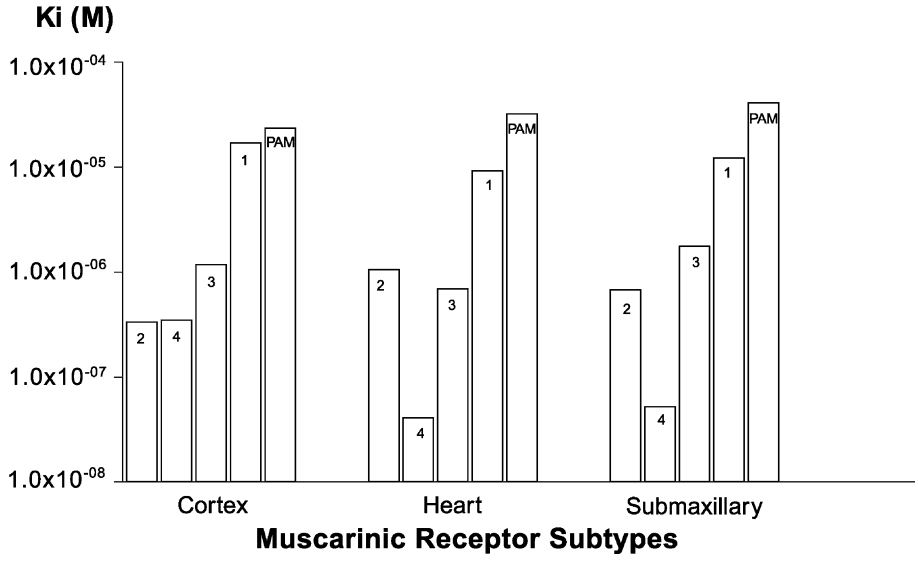


Fig. 1. K_i values of aldoximes **1**–**4** and pralidoxime (PAM) for muscarinic receptors in rat brain (cerebral cortex, predominantly M_1), heart (M_2) and submaxillary glands (M_1/M_3). **1**, **2**, **3**, **4** refer to the *O*-methyl, *O*-benzyl, *O*-propynyl and *O*-butynyl derivatives of pralidoxime, respectively.

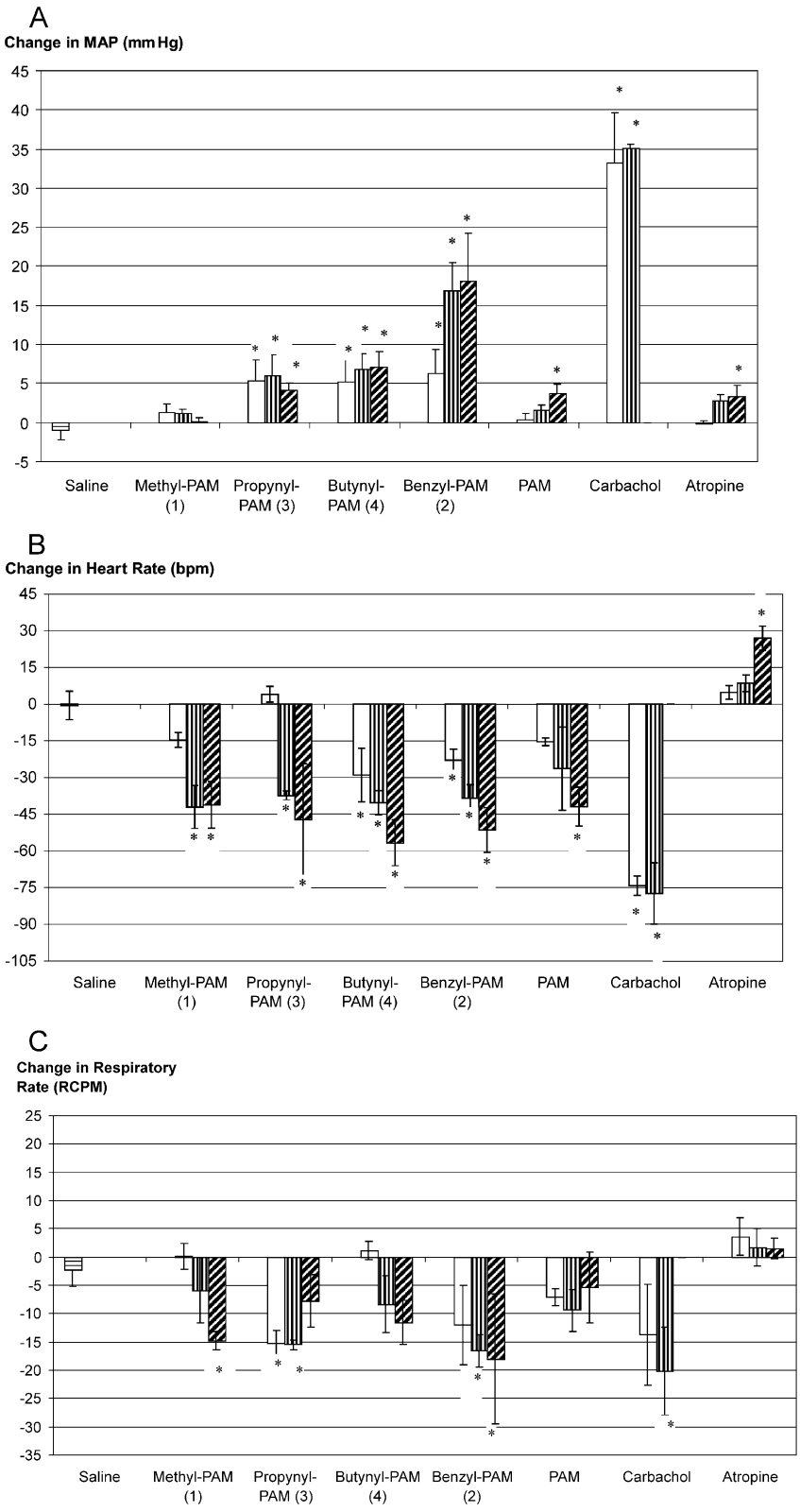


Fig. 2. Effects of aldoximes **1**–**4**, pralidoxime, carbachol and atropine on (A) mean arterial blood pressure (mm Hg); (B) heart rate (beats per minute); (C) respiratory rate (respiratory counts per minute) when administered i.c.v. into rats at doses of 0.09 μmol/kg □, 1.67 μmol/kg ▥, 4.50 μmol/kg ▨. Asterisk indicates response is significantly different ($P < 0.05$, unpaired single tailed *t*-test) from control responses in rats that received only saline via the same route.

oxotremorine M can be used to predict agonist efficacy at muscarinic receptors. Full agonists would have ratios greater than 100 while antagonists would normally give ratios ranging from 1 to 10. Intermediate values are indicative of partial agonism (Bromidge et al., 1995).

Table 2 gives the K_i values of the aldoximes for the inhibition of the binding of various tritiated ligands to different tissue homogenates. The aldoximes **1**–**4** exhibit consistently stronger binding affinities to the various muscarinic receptor subtypes than pralidoxime. The greatest increases in binding affinity are seen for the muscarinic M_2 receptor subtype of cardiac tissue. For example, *O*-but-2-ynyladoxime **4** has a thousand-fold stronger binding affinity than pralidoxime on this tissue. Smaller increases in binding affinity are observed for the muscarinic M_1 receptor subtype of cortical tissue. Another interesting observation relates to the relative binding affinities of the aldoximes for the various subtypes. For all three muscarinic receptor subtypes, binding affinity increases with increasing size of the *O*-substituent, up to the four carbon chain of the *O*-but-2-ynyl derivative **4** (Fig. 1).

Unlike pralidoxime and the aldoximes that have little or no selectivity for the muscarinic M_1 and M_2 receptor subtypes, **4** has an eight-fold selectivity for the muscarinic M_2 receptors of cardiac tissue. However, it has a smaller or no selectivity for the other receptor subtypes.

The ratio of K_i values for the displacement of [^{3}H] pirenzepine and [^{3}H] oxotremorine M (PZ/Oxo-M) is used to predict the pharmacological profile of the aldoximes. The ratios of the aldoximes (including pralidoxime) are less than 5, suggesting that these compounds are muscarinic receptor antagonists.

Confirmation of antagonist activity in these compounds was carried out by functional assays on the isolated guinea pig ileum which has a large population of muscarinic receptors. The aldoximes, including pralidoxime, depressed acetylcholine-induced contraction of the ileum and displaced the cumulative acetylcholine dose response curve to the right in a nonparallel manner. The effect was concentration dependent and not surmountable even at the highest concentration of acetylcholine. This suggests that the aldoximes **1**–**4** are noncompetitive muscarinic receptor antagonists. In contrast, atropine displaced the acetylcholine dose response curve to the right of the control curve in a parallel manner, which is indicative of competitive inhibition. The antagonistic effects of the aldoximes were quantified by their pK_B values ($-\log K_B$, where K_B is the noncompetitive dissociation constant, Table 2). The following rank order of inhibition was observed: **4** > **2** > **3** > **1** > pralidoxime. Pearson correlation gave an excellent agreement between the binding affinities of the aldoximes (based on pK_i values for each of the three subtypes) and the pK_B values obtained from the functional assay on the guinea pig ileum ($P < 0.01$). The *O*-substituted aldoximes are only slightly stronger cholinesterase inhibitors than pralidoxime, with IC_{50} values in the millimolar range (Table 2).

Rats were pretreated (i.c.v.) at three different dose levels (0.09, 1.67 and 4.5 μmol/kg) of pralidoxime and derivatives **1**–**4** before soman administration. The effects of these drugs on the mean arterial pressure, heart rate and respiration rate at each dose level are given in Fig. 2. Carbachol and atropine are control drugs investigated at similar dose levels. Fig. 2A shows the effects of these drugs on mean arterial blood pressure. A general trend of dose-dependent increase in blood pressure is evident among the test compounds. The pressor effects of the *O*-benzyl derivative **2** is notable and it causes higher increases in blood pressure than the other compounds at comparable doses. Increases in blood pressure were also observed with carbachol and atropine, with carbachol having a stronger pressor effect.

Fig. 2B shows the effects of the test compounds on heart rate. All the compounds caused a dose dependent slowing in heart rate, similar to that observed with pralidoxime. The acetylcholine receptor antagonist atropine caused a dose-dependent increase in heart rate, in contrast to the acetylcholine receptor agonist carbachol which is associated with a strong dose-dependent decrease in heart rate.

Fig. 2C shows the effects of the test compounds on the respiration rate of rats at the different dose levels. A decrease in respiration rate was observed for all compounds, with the greatest depression observed for the *O*-benzyl derivative **2**. Carbachol but not atropine causes a decrease in respiration rate.

Rats treated with soman (s.c., 0.9 LD_{50}) showed an increase in mean arterial pressure, a decrease in heart rate and respiratory depression (depicted as the saline bar in Fig. 3A–C).

Pretreatment with atropine at all dose levels (0.09, 1.67 and 4.5 μmol/kg) did not protect against the soman-induced pressor effects, as blood pressure remained at about the same levels as in the control soman-treated rats. This is also true for the other aldoxime derivatives, but pralidoxime and the *O*-benzyl derivative **2** caused blood pressure to rise significantly on soman administration (Fig. 3A).

Soman at 0.9 LD_{50} caused a slowing of heart rate by about 250 beats per min (bpm). Pretreatment with atropine (4.5 μmol/kg) had a beneficial effect of overcoming the bradycardia to some extent, which may be attributed in part to the anticholinergic effects of atropine (Fig. 3B). Since pralidoxime and its derivatives intrinsically cause bradycardia, one would expect little or no change in soman-induced bradycardia following pretreatment with these drugs. This is indeed observed (Fig. 3B). Exceptions are the *O*-benzyl derivative **2** and carbachol, both of which gave significant protection against bradycardia at 4.5 and 1.67 μmol/kg, respectively ($P < 0.05$).

Soman (0.9 LD_{50}, s.c.) caused respiration to be depressed by about 30 counts per minute (cpm) in rats. Significant protection against respiratory depression was observed when the animals were pretreated with atropine, the *O*-propynyl **3** and *O*-butynyl **4** derivatives at the highest dose of 4.5 μmol/kg ($P < 0.05$).

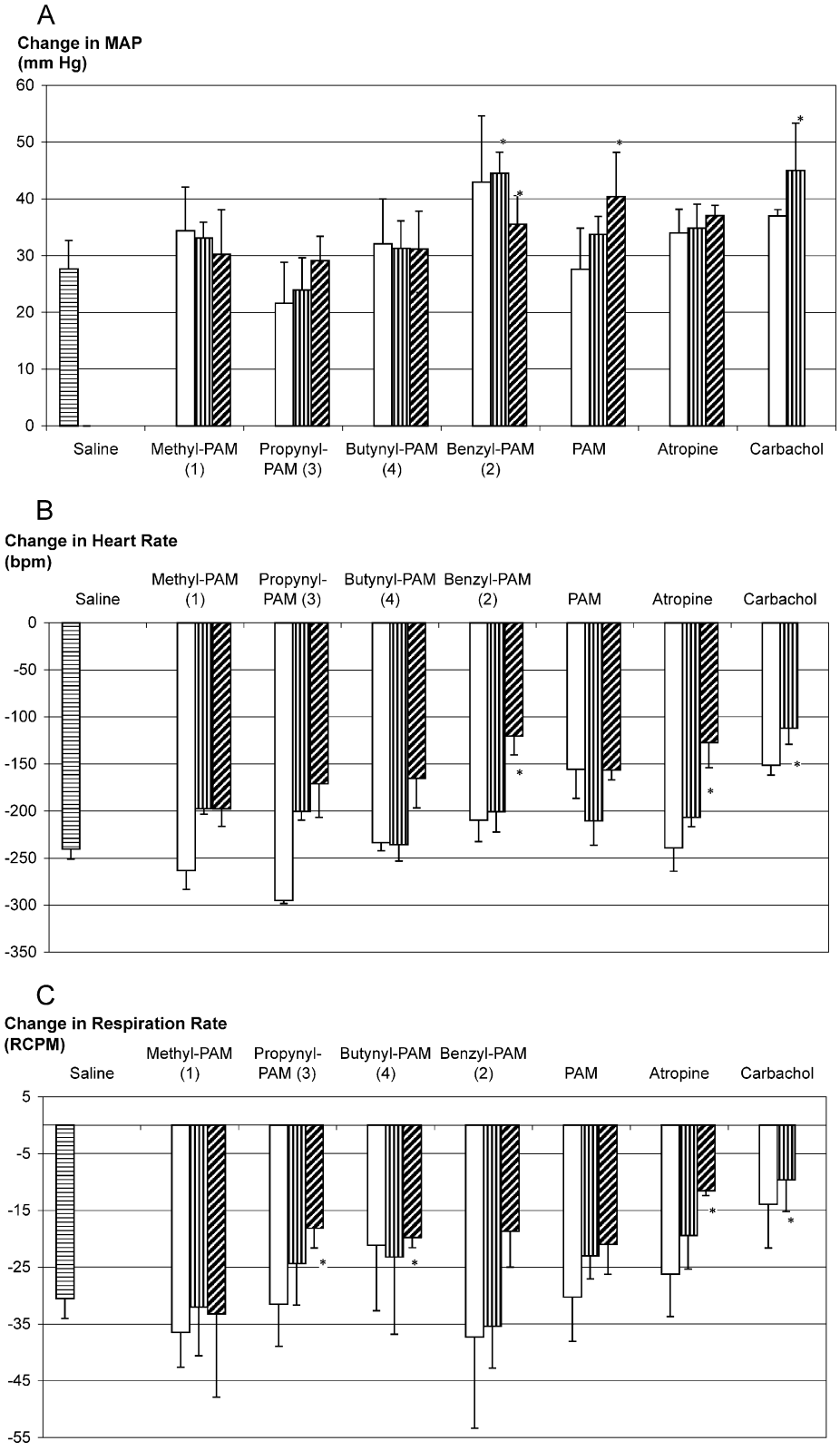


Fig. 3. Effects of aldoximes **1**–**4**, pralidoxime (PAM), carbachol and atropine on soman-induced changes in (A) mean arterial blood pressure (mm Hg); (B) heart rate (beats per minute); (C) respiratory rate (respiratory counts per minute) when administered i.c.v. into rats at doses of 0.09 μmol/kg □, 1.67 μmol/kg ▥, 4.50 μmol/kg ▧. The animals ($n = 3$ for each dose of drug) were pretreated with the above mentioned drugs and the parameter monitored for 30 min (as shown in Fig. 2). Soman (subcutaneous, $0.9 \times LD_{50}$) was then administered and the same parameters monitored for a further 4 h. Drugs are dissolved in 10% v/v dimethylsulfoxide (DMSO) in 0.9% (w/v) saline. Asterisk indicates significant differences ($P < 0.05$, unpaired single tailed *t*-test) from control rats that received only saline via i.c.v. before soman administration.

4. Discussion

In contrast to pralidoxime, the *O*-substituted derivatives are large lipophilic molecules, with size and lipophilicity increasing in the order **1** < **3** < **4** < **2** as the side chain is increased from methyl to benzyl. There was a good correlation between size of the molecule and the lipophilicity of the side chain. *O*-Substitution would also result in a loss of the H bond donating property of the oxime although the prop-2-ynyl aldoxime **3**, with its terminal alkynyl linkage may function as H-bond donor via its weakly acidic alkynyl H.

O-Substitution of pralidoxime resulted in important changes in the pharmacological profile of the compounds. The *O*-substituted derivatives are unlikely to function as cholinesterase reactivators. Pralidoxime is itself a selective reactivator and has no effect on soman or tabun-inhibited acetylcholinesterase due to the rapid aging of the inhibited enzyme.

All four *O*-substituted derivatives showed greater antimuscarinic activity than pralidoxime (pK_B decreasing in the order of **4**>**2**>**3**>**1**>pralidoxime). Like pralidoxime, they are noncompetitive inhibitors of acetylcholine-induced contraction of the guinea pig ileum.

The strong antagonist activity of the *O*-but-2-ynyl aldoxime **4** coincides with its strong binding affinity to the muscarinic receptors of cardiac tissue (mainly M_2) and submandibulary glands (mix of M_1 and M_3). **4** is also the only derivative to demonstrate selectivity in its binding affinity (10-fold selectivity for muscarinic M_2 cardiac receptors over muscarinic M_1 receptors of brain).

The *O*-butynyl side chain present in **4** has been associated with good muscarinic affinity or selectivity from earlier studies. For example, of the nine tropinone *O*-alkynyl oximes investigated as muscarinic ligands, the *O*-but-2-ynyl derivative demonstrated the greatest selectivity for muscarinic M_1 receptors and was subsequently shown to demonstrate cholinergic agonist properties (Xu et al., 1998). Similarly, *O*-but-2-ynyloxime of quinuclidin-3-one had a pirenzepine/oxotremorine M ratio of 120 and was shown to exert mnemonic effects in mice in the swimming escape task (Somanadhan et al., 2002). In this study, the *O*-but-2-ynyl aldoxime **4** turned out to be a noncompetitive muscarinic receptor antagonist with submicromolar affinities for the muscarinic M_2 subtype and a modest muscarinic M_2 receptor selectivity. It should be added that the ring structure to which the oxime side chain is attached is equally important in deciding the final pharmacological profile. For example, the piperidinone *O*-but-2-ynyl oxime has only weak muscarinic binding affinity (Xu et al., 1998).

The *O*-benzylderivative **2** has been reported to be a weak competitive inhibitor of acetylcholinesterase (IC_{50} 0.1 mM) (Powers et al., 1993). This was confirmed in the present study where its IC_{50} for inhibition of electric eel acetylcholinesterase was found to be 0.77 mM. The larger size and lipophilicity of **2** may have contributed to its stronger anticholinesterase activity. Comparing the anticholinesterase and anticholinergic effects of the *O*-benzyl **2**, *O*-propynyl **3** and *O*-butynyl **4** derivatives, one would expect the stronger anticholinergic effects of these compounds to prevail over their weaker anticholinesterase activities. This does not appear to be so. When administered to rats, the effects of the aldoxime derivatives on heart rate, respiration and to a lesser extent, blood pressure, are more closely aligned to the acetylcholine receptor agonist carbachol than the antagonist atropine. This may be due to the cholinesterase inhibitory activities of these compounds. Although these compounds are weak cholinesterase inhibitors, they are administered directly into the ventricle of the rat brain whose volume has been estimated to be only 1 cm^3 (Tinsley et al., 2001). Thus, the final concentration of drug in the brain is quite high. For example, at a dose of 4.5 μmol/kg, calculations based on the estimated brain volume shows that a concentration of 89 mM can be attained in the brain, which is well within the range of the anticholinesterase activities of these compounds. Therefore, the aldoxime derivatives should not be expected to behave solely as anticholinergic agents, but can exhibit a mix of acetylcholine receptor agonist and antagonist effects. Indeed, the effects of pralidoxime and its *O*-substituted aldoximes on blood pressure, heart rate and respiratory rate are more similar to that of carbachol than atropine, suggesting that the balance is tilted towards a net acetylcholine receptor agonistic effect.

Organophosphates are known to exert potentially harmful cardiovascular effects that are both centrally and peripherally mediated. Paraoxon and soman produced a marked and sustained increase in blood pressure in conscious unrestrained rats (Bataillard et al., 1990). Soman is known to induce myocardial degeneration and necrosis (Van den Beukel et al., 1997). In this study, rats administered subcutaneous soman at 0.9 LD_{50} developed bradycardia, hypertension and a reduction in respiratory rate. Bradycardia may be due to a cholinergic effect. Similarly, hypertension can be traced to the central effects of acetylcholine, although the cholinergic influence on the cardiovascular system is much less when compared to sympathetic control. Respiratory failure is the primary cause of fatalities in organophosphate poisoning (Taylor, 2001). Central respiratory depression and peripheral cholinergic effects contribute collectively to respiratory embarrassment and eventual death.

Pretreatment with atropine is seen to offer some protection against soman-induced bradycardia and respiratory depression but not against hypertension. No protection was evident after pretreatment with pralidoxime, but some of the aldoximes did protect against soman-induced bradycardia (*O*-benzyl derivative **2**) and respiratory depression (*O*-propynyl **3** and *O*-butynyl **4** derivatives). Interestingly, pretreatment with carbachol also reduced soman-induced bradycardia and respiratory depression and at a lower dose level.

The present study shows that some *O*-substituted aldoximes related to pralidoxime can offer protection against soman-induced bradycardia and respiratory depression. Their

protective effects are comparable to atropine and superior to pralidoxime, which offered no protection at similar doses. However, it is evident that the limited protective effects seen with these pralidoxime derivatives (**2**, **3**, **4**) do not stem exclusively from their anticholinergic effects. More likely, the ability of these aldoximes to alleviate bradycardia and respiratory depression may be the outcome of compensatory mechanisms, of which the acetylcholine receptor agonist and antagonist effects of these drugs may be contributory.

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