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THE INTERACTION OF BIS-PYRIDINIUM OXIMES WITH MOUSE BRAIN MUSCARINIC RECEPTOR

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Abstract—The bispyridinium oximes toxogonin [*N,N'*-oxydimethylene bis (pyridinium 4-aldoxim) dichloride] and its structural analogs HS-3, HS-6, HI-6 and MMB-4, and the bispyridinium salt SAD-128, which serve as antidotes to certain types of organophosphorus poisoning, bind competitively to mouse brain muscarinic receptors. This was determined *in vitro* employing the potent and specific muscarinic antagonist ^{3}H-4NMPB (^{3}H-4-*N*-methyl piperidyl benzilate). All the bispyridinium compounds also exerted a mild anti-acetylcholine activity ($K_d = 10^{-4}$–10^{-5} M) measured physiologically in the guinea pig ileum, which correlated well with the dissociation constants obtained from binding studies with mouse brain homogenate. The most potent muscarinic blocker was SAD-128 ($K'_d = (7.1 \pm 1.2) \times 10^{-6}$ M for whole mouse brain), whose remarkable therapeutic action against soman intoxication may be partly attributed to this antimuscarinic activity.

The binding data are best fitted by a competitive model, and the deviation from the law of mass action observed here may be related either to the heterogeneity of muscarinic receptors in the mouse brain or to nonequivalency of the number of binding sites for bisquaternary pyridines and 4-NMPB.

It was already established in the 1950's [1, 2] that mono- and bispyridinium oximes such as 2-PAM-Cl (2-pyridine-aldoxime methyl chloride) and toxogonin [*N,N'*-oxydimethylene bis (pyridinium 4-aldoxime) dichloride], which serve as potent reactivators of inhibited acetylcholinesterase (AChE), could be used as antidotes against organophosphorus poisoning. However, these oximes (in combination with atropine) proved to be ineffective in protecting against soman (*O*-pinacolyl methylphosphonofluoridate) and other organophosphates that produce organophosphoryl-AChE conjugates which undergo rapid aging [3]. However, respiratory failure and neuromuscular blockade caused by soman in mice were successfully treated using atropine together with certain bispyridinium compounds HS-3, HS-6 and HI-6 [3], first synthesized by Hagedorn *et al.* (see ref. [4]).

It was later shown [5] that HS-6 administered to atropinized, anesthetized, soman-intoxicated rats delayed respiratory failure by one hour or more, and that HI-6 caused functional recovery of neuromuscular transmission in rats even when given one hour after soman [6]. The bisoxime HS-3 increased the rate of survival in dogs [7]. However, it was also noted that protection against soman could be obtained with SAD-128, a bisquaternary analogue of toxogonin in which both oxime groups are replaced by tertiary butyl groups [3].

The therapeutic effect of these bispyridinium derivatives could not be attributed to reactivation of the phosphorylated cholinesterase [6]. However, HS-6 and SAD-128 reduced the aging rate of somanylyl-AChE *in vitro* [8] and showed some parasympatholytic activity [9, 10]. It was suggested that both these activities might contribute to the protective potency of these oximes [8–10].

Since *in vivo* studies have shown that the quaternary oximes are capable of penetrating the blood brain barrier [6, 11–14], it is not unlikely that their protective efficacy may be partly related to their interaction with the cholinergic receptor in the brain. It has recently been shown that the potent labeled muscarinic antagonist ^{3}H-4-*N*-methyl piperidyl benzilate (^{3}H-4NMPB) is a useful tool for determining the characteristics of muscarinic receptor in the central [15–18] and peripheral [19, 20] nervous systems. Other potent labeled muscarinic antagonists have also been employed for this purpose [21–23]. In this study we used ^{3}H-4NMPB as a tool to demonstrate that the bisquaternary derivatives toxogonin, HS-3, HS-6, HI-6, MMB-4 and SAD-128 bind specifically to the muscarinic receptor in mouse brain. The potency of binding of the various antidotes was found to correspond well to their efficacy as antagonists of the muscarinic receptor as assayed physiologically in the guinea pig ileum.

MATERIALS AND METHODS

Materials

^{3}H-*N*-methyl-4-piperidyl benzilate (^{3}H-4NMPB) (55.4 Ci/mmole) was prepared and tested for purity as described earlier [15, 16].

Toxogonin, HS-3 [1-(2-hydroxyiminoethyl pyridinium) 1-(4-hydroxyiminoethyl-pyridinium)-dimethylether], HS-6 [1-(2-hydroxyiminoethyl-pyridinium) 1-(3-carboxy amido-pyridinium) dimethylether], HI-6 [1-(2-hydroxyiminoethyl-pyridinium) 1-(4-carboxyamido pyridinium) dimethylether], SAD-128 [bis (1.1'-(4-tertbutyl pyridinium) dimethylether] and MMB-4 [bis-(4-hydroxyiminoethyl-pyri-

dinium) methane] were prepared according to the methods described in ref. [4].

Methods

Effects on isolated organ. The acetylcholine-induced contraction of isolated guinea pig ileum was measured as described elsewhere [24]. Dose ratios and dissociation constants were calculated from the dose–response curves for acetylcholine in the presence of different concentrations of the drug tested, according to Arunlakshana and Schild [25].

Competition binding experiments. Binding assays were performed using ^{3}H-4NMPB and mouse brain homogenates, according to the technique described in detail in previous reports [15, 16]. Competition binding studies were carried out in two complementary types of experiments: (1) the binding of ^{3}H-4NMPB at fixed concentration (2.0 nM) was measured in the presence of different concentrations of the unlabeled drugs; (2) the binding of ^{3}H-4NMPB at different concentrations was measured in the presence of fixed concentrations of the unlabeled drugs.

The data were analysed assuming a model by which the labeled and unlabeled ligands compete for the same receptor binding sites, as previously described [15, 16].

Samples were assayed for radioactivity by liquid scintillation spectrometry (Packard Prias Model PL), and corrections for quenching were made by using a quench curve based on the external standard ratio method, using standard tritiated water and toluene (Packard). Specific binding was defined as the total binding minus binding in the presence of 5×10^{-5} M unlabeled 4-NMPB or atropine.

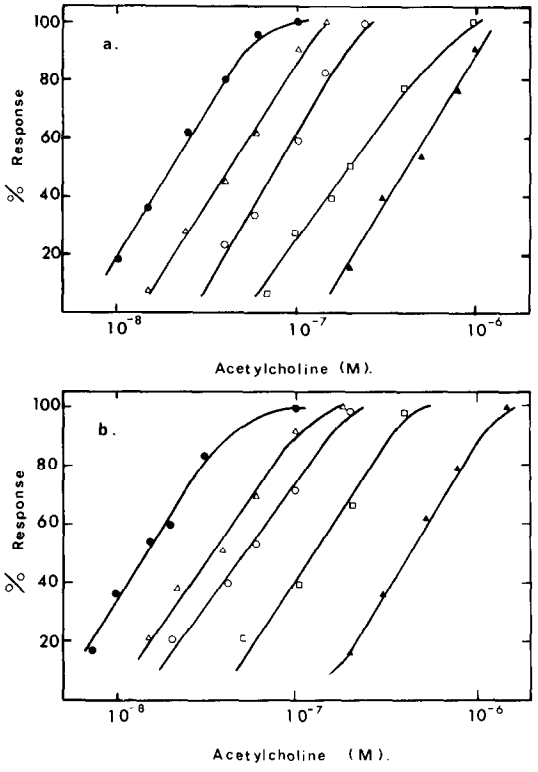

Fig. 1. Antagonism of SAD-128 (panel a) and HS-3 (panel b) to the contractile response of guinea pig ileum to acetylcholine (●). SAD-128 concentrations were (M): 1×10^{-5} (△); 3×10^{-5} (○); 1×10^{-4} (□), 2.5×10^{-4} (▲). HS-3 concentrations were (M): 5×10^{-5} (△); 1×10^{-4} (○); 2.5×10^{-4} (□); 1×10^{-3} (▲). Ordinate: response (%).

RESULTS

The bis-pyridinium compounds tested exerted a mild anti-acetylcholine activity relative to that of atropine as measured by the contractile response of the smooth muscle. At the concentration range investigated (10^{-5}–10^{-3} M), the drugs did not provide contraction of the muscle preparation. Representative dose–response curves of acetylcholine in the presence of SAD-128 and HS-3 are given in Fig. 1. The parallel shift of the dose–response curves of acetylcholine in the presence of the drugs, as well as the surmountable nature of the antagonism, indicate that SAD-128 and HS-3 acted as competitive antagonists. Similar results were obtained with all the drugs tested. The inhibitory effect curves of the bisquaternary pyridins plotted according to Arunlakshana and Schild [25] yielded a straight line with

Table 1. Apparent dissociation constants for bisquaternary pyridines determined in the guinea pig ileum and mouse brain

Drug	Guinea pig ileum Ka^* (M)	Brain $K'd^\dagger$ (M)	n_h
SAD-128	$(1.0 \pm 0.7) \times 10^{-5}$	$(7.1 \pm 1.2) \times 10^{-6}$	0.8
HS=3	$(3.2 \pm 1.2) \times 10^{-5}$	$(2.4 \pm 1.1) \times 10^{-5}$	0.84
Toxogonin	$(6.2 \pm 1.4) \times 10^{-5}$	$(4.2 \pm 2.0) \times 10^{-5}$	0.85
HS-6	$(1.0 \pm 0.8) \times 10^{-4}$	$(2.9 \pm 1.9) \times 10^{-4}$	0.80
HI-6		$(1.9 \pm 0.9) \times 10^{-4}$	0.88
MMB-4	$(3.2 \pm 2.0) \times 10^{-4}$	$(2.0 \pm 1.3) \times 10^{-4}$	0.92

* Ka values, expressed as the mean ± S.E.M., were calculated according to Arunlakshana and Schild [25].

† $K'd$ values, expressed as the mean ± S.E.M., were calculated assuming a competitive interaction between ^{3}H-4NMPB and the unlabeled drugs as described elsewhere [16].

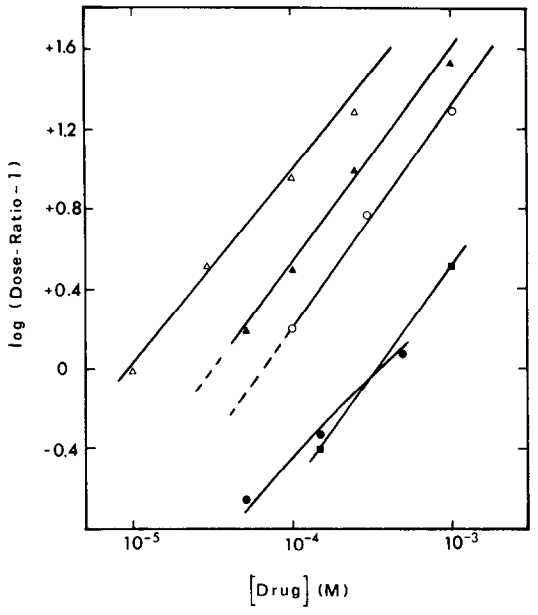

Fig. 2. Inhibitory effect curves of the bisquaternary pyridines: SAD-128 (△); HS-3 (▲), toxogonin (○); MMB-4 (●); HS-6 (■), in the guinea pig ileum preparation plotted according to Arunlakshana and Schild [25]. Dose ratio is the ED_{50} ratio of acetylcholine in the presence and in the absence of the antagonist. Abscissa: Antagonist concentrations (M).

a slope of 45°, except for MMB-4 (Fig. 2), thus again indicating competitive antagonism. The dissociation constants (K_d) evaluated by the method of Arunlakshana and Schild [25] are summarized in Table 1.

The binding characteristics of the bisquaternary pyridines to the muscarinic receptors in mouse brain were examined by competition binding experiments using the potent and specific labeled muscarinic antagonist ^{3}H-4NMPB [15, 18]. Figure 3a demonstrates inhibition of specifically bound ^{3}H-4NMPB (fixed concentration) by various concentrations of the bisquaternary pyridines; the specific binding of the labeled antagonist is virtually suppressed by the unlabeled drugs. The data in Fig. 3a, when replotted according to Hill [26], yield straight lines with slopes of 0.8–0.9 (Fig. 3b, Table 1), indicating that the binding curves approach but do not exactly resemble simple mass action curves. However, the apparent dissociation constants measured in the binding experiments are similar to those measured by the antagonism to acetylcholine-induced contraction of guinea pig ileum (Table 1).

The nature of the binding of the bisquaternary pyridines was further investigated through binding experiments in which different concentrations of the labeled antagonist were used. Figures 4 and 5 represent double reciprocal plots for receptor binding of ^{3}H-4NMPB in the presence of various fixed concentrations of toxogonin and SAD-128, respectively. Intersection of all curves at the point 1.0 on the $1/\bar{y}$ axis shows that the competing bound toxogonin or SAD-128 could be completely replaced by ^{3}H-4NMPB at sufficiently high ^{3}H-antagonist concentrations. The straight lines show only a single class of noninteracting binding sites for toxogonin or SAD-128. A plot of the apparent dissociation constants (calculated from the slopes in Figs. 4 and 5) as a function of the concentration of toxogonin or SAD-128 (Figs. 3 and 4, inserts) did not give a linear relationship. If a simple competition model is assumed, this would indicate that the number of binding sites for toxogonin or SAD-128 differs from that for ^{3}H-4NMPB [27]; otherwise a ternary complex comprising bisquaternary pyridine–receptor–^{3}H-4NMPB should be assumed [16, 27].

DISCUSSION

It has been found previously that HS-6 blocks the ganglion stimulating effect of excitatory nicotinic agonists [28] and also blocks the neuromuscular junction [29]. Weak antimuscarinic activity measured physiologically with guinea pig ileum has been attributed to toxogonin [30], HS-6 [9] and SAD-128 [10], and it was suggested that the bispyridinium oximes are noncompetitive antagonists [9, 30]. The bisquaternary pyridines tested in the present study exert antimuscarinic activity, as shown by their blocking effect on the acetylcholine-induced response of the guinea pig ileum and by their inhibition of the binding of the specific muscarinic antagonist 4-NMPB [15] to mouse brain homogenates. In the light of previous findings [26, 27], it is not unlikely that some of the bisquaternary oximes might also block central nicotinic receptors, a possibility which we are currently investigating.

As shown in the case of other muscarinic antagonists [15, 16, 20, 21], there is excellent agreement between the apparent dissociation constants determined by employing these two methods. The most potent bispyridinium antagonists are SAD-128, HS-3 and toxogonin, while HS-6, HI-6 and MMB-4 are less potent. These results correlate well with previously reported findings [9, 10, 30]. Hence, the most active antagonists are the dimethylether bisquaternary pyridines having a substituent in position 4 of the pyridine ring, provided that the substituent at this position is not a carbamide group (HI-6). On the other hand, it is not essential that it should be an oxime group, since SAD-128 has two tertiary-butyl groups instead of the oxime moiety.

The parallel shift of the dose–response curves for ACh in the presence of the bisquaternary pyridines, as well as the 45° slope of the inhibitory effect curves, point to competitive antagonism. Similar results were obtained by Kuhnen-Clausen for toxogonin [9, 30] and other bisquaternary pyridines at concentrations up to 1 mM. At higher toxogonin concentrations (up to 10 mM) this author has shown that the inhibitory effect curves do not follow a simple mass action law [30].

The binding of the bisquaternary pyridines to mouse brain muscarinic sites cannot be explained by a simple mass action model either. However, several lines of evidence support the assumption of purely competitive binding between ^{3}H-4NMPB and each of the bisquaternary pyridines: (1) The drugs inhibit almost all the binding of 2.0 nM ^{3}H-4NMPB. Under these experimental conditions the binding of the labeled drug in the absence of unlabeled drug yields

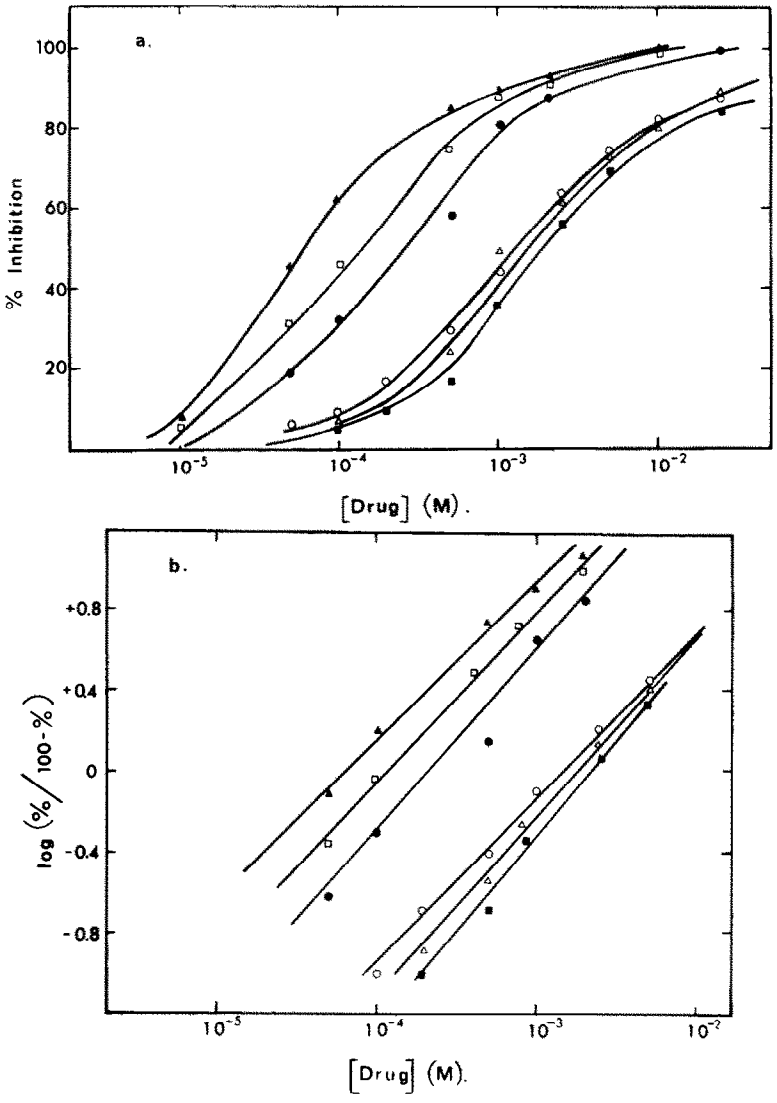

Fig. 3. Competition binding of bisquaternary pyridines and 2.0 nM ^{3}H-4NMPB in mouse brain homogenate. Panel a: Samples (0.05 ml) were incubated at 25° for 30 min in 2 ml modified Krebs solution containing 2.0 nM ^{3}H-4NMPB and the unlabeled drugs at the concentrations indicated. Specific binding was determined as described in Methods. ^{3}H-4NMPB binding site concentration was 0.2 nM and the binding in the absence of unlabeled drug was 0.17 nM. Each point represents the average of triplicate determinations. (▲) SAD-128; (□) HS-3; (●) toxogonin; (○) HS-6; (△) HI-6; (■) MMB-4. Panel b: Hill plot of the same data. % refers to per cent inhibition.

an occupancy of more than 85 per cent. Therefore in the presence of the unlabeled drug most of the latter's binding occurs at the expense of ^{3}H-4NMPB binding; (2) The common intercepts of the double reciprocal plots indicate that the competing bound SAD-128 or toxogonin could be completely replaced by ^{3}H-4NMPB at sufficiently high concentrations; (3) The straight lines of the double reciprocal plots indicate that there is no possibility of cooperative binding of the bisquaternary pyridines; (4) Preliminary kinetic binding studies (unpublished data) have shown that the apparent association and dissociation rate constants of ^{3}H-4NMPB do not change in the presence of SAD-128 or toxogonin.

We conclude that the binding data presented are best fitted by a competitive model which describes the binding of the bisquaternary pyridines and the specific muscarinic antagonist 4-NMPB in mouse brain homogenate. The deviation from the law of mass action may be related either to the heterogeneity of muscarinic receptors in the mouse brain [17, 18] or to nonequivalency in the number of binding sites for the bisquaternary pyridines and for 4-NMPB. However, if the binding of ^{3}H-4NMPB and the bisquaternary pyridines is not mutually exclusive, that is, if the binding of the unlabeled drug does not coincide with the displacement of ^{3}H-4NMPB from its receptor binding sites, the existence of a ternary

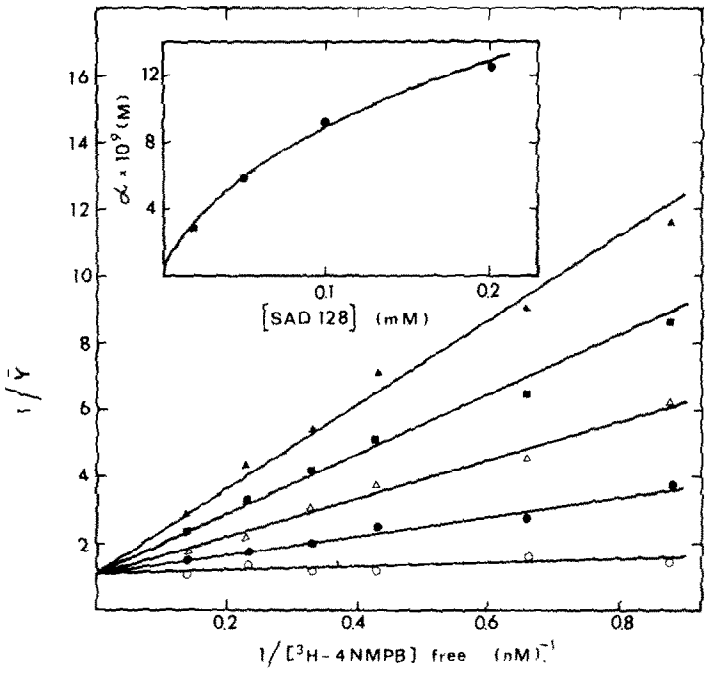

Fig. 4. Double reciprocal plots of ^{3}H-4NMPB binding *in the presence of SAD-128.* Samples were incubated as described in Fig. 3 with various concentrations of ^{3}H-4NMPB *in the absence* (○) and in the presence of SAD-128 at concentrations (mM): 0.02 (●), 0.05 (△), 0.1 (■) *and* 0.2 (▲). Specific binding was determined as described in Methods. *Each point represents the* average of triplicate determinations. $\bar{Y}$ is the fractional occupancy of the *receptors by* ^{3}*H-4NMPB where the* binding sites concentration was 0.2 nM. Insert: The apparent dissociation constants (α) calculated *from the slopes* of the double reciprocal plots, plotted as a function of SAD-128 concentration.

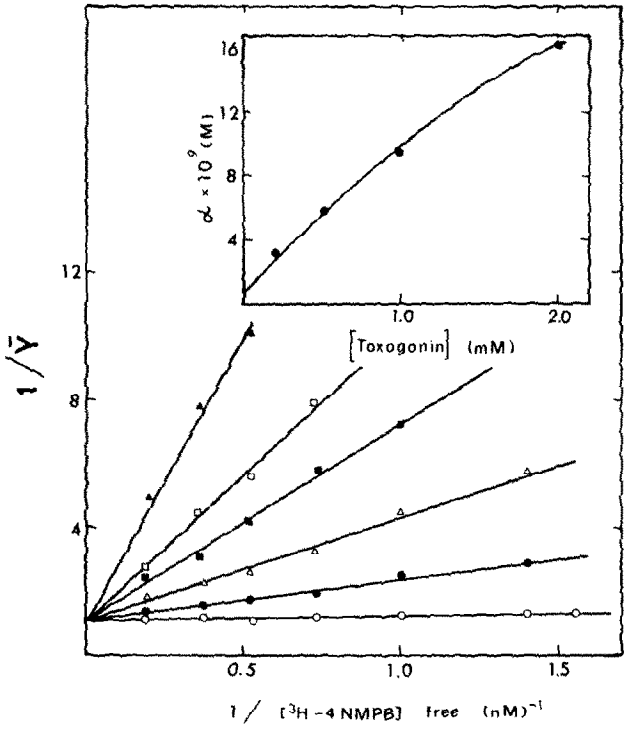

Fig. 5. Double reciprocal plots of ^{3}H-4NMPB binding *in the presence of* toxogonin. Samples were incubated as described in Fig. 3 with various concentrations of ^{3}H-4NMPB *in the absence* (○) and in the presence of toxogonin at concentrations (mM): 0.05 (●), 0.2 (△), 0.5 (■), *1.0* (□) *and* 2.0 (▲). Specific binding was determined as described in Methods. Each point represents the average of triplicate determinations. $\bar{Y}$ is the fractional occupancy of the receptors by ^{3}H-4NMPB, where the binding sites concentration was 0.20 *nM. Insert:* The apparent dissociation (α), calculated from the slopes of the double reciprocal plots, plotted as a function of toxogonin concentration.

complex should be assumed [16]. In this case a kind of allosteric mechanism may be considered, as has been previously suggested [30].

These studies of binding to mouse brain muscarinic receptors support the hypothesis that some of the bispyridinium compounds, especially SAD-128, may exert their therapeutic action by blocking central muscarinic receptors. The concentrations of the bisquaternary pyridines used in this study are relevant to their therapeutic dosage. It is noteworthy that their low affinity towards the muscarinic receptors may be compensated by their relatively high levels in the blood (50–500 μM) [5, 28, 31]. It should be noted, however, that the bispyridinium compounds may exert different binding properties and physiological actions under conditions of organophosphorus intoxication.

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