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BINDING OF SOMAN ANTIDOTES TO ACETYLCHOLINE RECEPTORS

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Abstract—It has been reported that several bis-quaternary compounds not necessarily having an oxime function can be used to treat soman poisoning in mice. The mechanism for this protection is not clear, but it has been proposed that such compounds may act by blocking muscarinic or nicotinic acetylcholine receptors. We have tested thirty-four compounds for muscarinic binding activity, using displacement of tritiated 3-quinuclidinyl benzilate (QNB) as a criterion, and thirty-two compounds for nicotinic binding based on inhibition of alpha-bungarotoxin (BGT) binding. Only sixteen of these compounds were able to displace QNB from rat brain membranes, and only ten of them affected BGT binding. With one exception, all of the effective compounds belonged to a series of bis-pyridinium compounds that are similar in structure to SAD-128. The binding affinities to muscarinic receptors of all these compounds were low compared to atropine. Some of the compounds bound to nicotinic receptors with affinities approaching that of *d*-tubocurarine. However, there was not a direct correlation between binding affinity and their reported efficacy against soman.

The lethality of organophosphorus anticholinesterases is presumably due to the inhibition of acetylcholinesterase (AChE) and the resulting accumulation of high concentrations of acetylcholine in the cholinergic synaptic cleft [1]. If AChE activity can be restored, as it can after poisoning with common insecticides, such as paraoxon, and certain, more toxic organophosphates, such as di-isopropylphosphorofluoridate (DFP) and methylisopropylphosphonofluoridate (sarin), then it is possible to raise the LD_{50} of such anticholinesterases by as much as two orders of magnitude [2, 3]. However, when AChE is inhibited by methylpinacolylphosphonofluoridate (soman) and certain other related compounds, it subsequently "ages" very rapidly with a half-time ($T_{1/2}$) of a few minutes *in vitro*, and it is not possible to restore the AChE activity [4, 5]. Animals poisoned with such aging anticholinesterases nevertheless do respond, to a limited extent, to treatment with bis-quaternary oximes, such as HS-3, HS-6 and HI-6 [5], as well as with certain other quaternary compounds, such as SAD-128, that do not possess an oxime function [5–8]. The therapeutic effect of these compounds is probably not due to reactivation of the phosphonylated AChE because once aging occurs cholinesterase cannot be reactivated. This observation implies that such compounds have another function not necessarily related to AChE reactivation. Schoene [7] proposes several possible mechanisms for anti-soman effectiveness of bis-pyridinium compounds, including inhibition of cholineacetyl transferase, reactivation of AChE, protection of AChE against soman, and retardation of aging of soman-inhibited AChE. He has shown [9] a positive correlation between reversible inhibition constants of these compounds and effectiveness for soman treatment, but there are discrepancies that make this explanation questionable as the primary protective mechanism. Although SAD-128 [10], as well as several other bis-pyridinium compounds [11], does inhibit the aging of cholinesterase, the rate is slowed only slightly, and it does not seem likely that the inhibition of the aging rate is adequate to explain the protection this compound affords against intoxication by soman [11].

Therefore, it appears that SAD-128 and other bis-pyridinium compounds are effective in the treatment of soman poisoning by a mechanism other than the reactivation of AChE. The effectiveness of these compounds could be due to either agonist or antagonist activity on the cholinergic receptors in the brain, the periphery, or the ganglia. The receptor binding studies of Kuhnen-Clausen *et al.* [12], in which they measured the binding of several "H-Oximes" (bis-quaternary pyridinium salts having an oxime function) to both nicotinic and muscarinic receptors, lend some support to this hypothesis. Although they were unable to find any correlation between nicotinic binding and antidotal activity, they did seem to demonstrate a relationship between antidotal effectiveness and muscarinic binding. The present experiments represent an attempt to extend those results by testing whether certain bis-quaternary compounds, some of which have been shown to be effective in the treatment of anticholinesterase poisoning, bind at the QNB binding site (presumably the agonist recognition site) of the muscarinic receptor or at the BGT binding site of the nicotinic receptor.

To evaluate the significance of those binding results, a number of other compounds that have been suggested as possible prophylactic or therapeutic compounds against soman were included. The prem-

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ise adopted here is that, if any compound is going to have a direct effect at the synapse, it would have to bind to the acetylcholine receptor with a low binding constant, that is, having a high binding affinity. Since direct measurements of binding would require a radioactive sample of high specific activity for each compound, displacement binding studies were chosen. In displacement binding, the binding site for the effector would coincide with that of known specific antagonists (ligands) and should displace them with some concentration close to the effective *in vivo* concentration. Since these are displacement binding studies, they only determine whether these compounds bind at the active site (the agonist-binding site) of the receptor. If the effectiveness of these compounds is due to binding at an allosteric site on the receptor that affects agonist but not antagonist binding, such binding might not be detected by this type of experiment. Some experiments similar to those described here have been done by Amitai *et al.* [13] using bis-quaternary oximes. They were able to demonstrate a positive correlation between muscarinic binding strength of the compounds they tested and their antidotal efficacy, using 4-methyl-4-piperidyl benzilate as the test ligand.

MATERIALS AND METHODS

Materials. The compounds P61, 62, 63, 64, 65, 66, 105, 106, 107, HH-38, 39, 54, 64, HY-10 and 3,3'-LuH-6 were provided by Dr. Klaus Schoene, Aerobiology Institute, Grafschaft, West Germany. SAD-128 was synthesized by Dr. Morton L. Mednick, formerly of this Institute, now retired. The phosphinates were obtained from Ash-Stevens, Detroit, MI. [^{125}I]α-bungarotoxin was obtained from either New England Nuclear or Amersham; [^{3}H]QNB was obtained from the Amersham Corp., Arlington Heights, IL. All other chemicals were of analytical grade and were obtained from commercial sources.

Torpedo membrane preparation. Membranes rich in nicotinic acetylcholine receptors were prepared by a modification of the method of Sobel *et al.* [14]. Five hundred grams of frozen tissue was partially thawed, and then sliced, minced, and suspended in 250 ml of 0.01 M phosphate buffer, 1 M in NaCl. The tissue was homogenized with five bursts of a polytron (Brinkmann, Westbury, NY) at setting eight, 15 sec at each burst with 15 sec of cooling between bursts. The suspension was centrifuged at 1000 g for 10 min and decanted through cheesecloth (saving the supernatant fraction); the pellet was homogenized again in 125 ml of the phosphate buffer. After centrifuging, the supernatant fraction was combined with that from the previous step and the pellet was discarded. The combined supernatant suspensions were centrifuged at 100,000 *g* for 45 min, and the supernatant fraction was discarded. The resulting pellet was suspended in the homogenizing buffer, again centrifuged at 100,000 *g* for 45 min, and the supernatant fraction discarded. The pellet was suspended in 0.01 M phosphate buffer, pH 7.4, containing 16% sucrose to a concentration of 1.5 pmoles of receptor per ml. This suspension was used directly for binding measurements. Preparations were stored at −90° for up to 4 months with no noticeable change in binding parameters.

Nicotinic binding assays. Ten microliters of a given concentration of the test ligand was added to triplicate samples of 100 μl of a suspension of torpedo membranes. At the same time, controls containing either 10 μl of 10 mM *d*-tubocurarine or 10 μl of phosphate-buffered saline (PBS), in lieu of test ligand, were also prepared. Each sample was diluted with 400 μl of PBS (final protein concentration 0.7 mg/ml) and incubated at room temperature for 5 min. Then 10 μl of radiolabeled BGT was added to each tube (final concentration 1 nM) and incubated at room temperature for 15, 30, 60, 120, or 240 min. After the measured time interval, each tube was diluted with 3 ml of PBS containing 1 mg bovine plasma albumin per ml and rinsed on a Whatman GF-B glass filter, using a vacuum filtration apparatus. The filter was then washed three times with 3 ml of PBS, transferred to a scintillation vial, and counted conventionally. Under these conditions, nonspecific binding (i.e. binding in the presence of 20 μM *d*-tubocurarine) ranged from 5 to 10% of total binding.

Each compound was initially screened by testing the reduction of binding of BGT to receptor membranes after a 10-min incubation in the presence of the test compound at 800 μM. If there was no change from the controls, the compound was judged "ineffective". If there was a change of 20% or less, the compound was considered "marginally effective". No further measurements were carried out on compounds that caused a change of 20% or less in the initial screen. If a change of more than 20% was observed, the initial rates of BGT binding were measured in the presence of several concentrations of the test compounds, as described above. When the initial rate constants are plotted against the log of the concentration of the competing ligand, a linear concentration–response line is obtained [15]. The concentration of competing ligand that reduced binding rate by 50% is defined as the IC_{50}.

Rat brain membrane preparation. The preparation of muscarinic membranes was similar to that of Yamamura and Snyder [16]. The caudate nucleus was removed from a decapitated rat, weighed, and then suspended in 10 ml of 0.32 M sucrose solution for each gram of wet weight. This was homogenized in a glass/Teflon Potter–Elvehjem homogenizer and then centrifuged at 1000 *g* for 5 min at 5°. The supernatant fraction was decanted and saved; the pellet was rehomogenized in 0.32 M sucrose and recentrifuged as described above. The supernatant fractions were then combined, homogenized with a polytron homogenizer (Brinkmann) at a setting of eight with five 5-sec bursts and 15 sec of cooling between bursts. The resulting suspension was then centrifuged at 100,000 *g* (Beckman L8-80) for 90 min. The pellets were suspended in distilled water to a concentration of 3–4 fmoles of muscarinic receptor sites per ml (based on radioactive ligand binding). While this is a rather elaborate preparation of material for binding studies, this procedure was used because the same membranes were used for other experiments that required the purer preparation. The radiolabeled

ligand used was [^{3}H]QNB, with a specific activity of 13 Ci/mmole. For later experiments, a preparation of 32 Ci/mmole was used, with completely comparable results. A standard filtration assay as described by Yamamura and Snyder [16] was used.

Muscarinic binding assays. Since the binding of QNB is completely reversible, it was possible to determine equilibrium competition binding constants for the test ligands. The protocol for the muscarinic ligand binding was as follows: 100 μl of the membrane suspension (10 mg/ml) containing 100 μl of 0.01 M phosphate buffer, pH 7.4, 10 μl of the test solution, and 10 μl of a 150 nM QNB solution were combined in a test tube and mixed thoroughly. To determine total binding and nonspecific binding, the test sample was replaced by saline or 100 μM scopolamine (to a final concentration of 3.3 μM scopolamine) respectively. We also demonstrated that unlabeled methyl QNB (Quarzan) could be used in lieu of scopolamine, with completely comparable results [17]. After incubation for 2 hr at room temperature or overnight at 4°, 3 ml of 0.01 M phosphate buffer, pH 7.5, was added and the suspension was rinsed on Whatman GF-B glass filters on a vacuum filtration apparatus with three 3-ml aliquots of PBS. The filter paper was then placed in a scintillation vial. Ten milliliters of Aquasol (New England Nuclear) was added, and the radioactivity was determined by scintillation spectrometry.

All compounds were screened first at a concentration of 800 μM, and those that suppressed the binding of the radiolabeled ligand by less than 20% were regarded as inactive. Binding curves using serial dilutions of the test compounds were run with compounds that displaced more than 20% of the radiolabeled ligand at 800 μM. The concentration at which 50% of the radiolabeled ligand was displaced is defined as the IC_{50} and its proportional to the K_D of the test compound. The data were analyzed on the Prophet system, using the public BINKIN 2 program, which will produce Scatchard, Lineweaver–Burk or Eadie–Wilkinson–Dixon plots, the latter one being recommended by Zivin and Waud [18] as the preferred analytical method for data of this type. The IC_{50} value reported here were calculated by the Eadie–Wilkinson–Dixon method and are the same as those calculated by the probit method, within the accuracy of the binding data.

RESULTS

Muscarinic binding. Twelve of the thirty-four compounds tested exhibited no inhibition of [^{3}H]QNB binding. They include mecamylamine, mikedamide, xylocaine, compound HH-54, isonitrosine (MINA), diacetylmonoxime (DAM), and all of the phosphinates. In addition, six compounds, HS-6, 3,3'-LuH-6, HH-38, HH-39, ketamine, and tiletamine, were able to displace [^{3}H]QNB only to a limited extent at 800 μM. Those that displaced the ligand at pharmacologically significant concentrations are shown in Table 1. Most of them (with a prefix P) belong to a homologous series of compounds synthesized by Schoene and his co-workers [7] and have been tested by them for efficacy in treatment of soman poisoning in mice [7].

In Schoene's screening procedure [7] using atropine-treated mice as test animals and measuring the ED_{50} against a LD_{95} soman challenge, P65 was the most effective compound of the series for the treatment of soman intoxication. P65 has a 6-carbon spacer between the nitrogen atoms. This spacing is comparable to that of hexamethonium, a well-known ganglionic, nicotinic antagonist. The most strongly bound of the test compounds was P107 (Table 1), a bis-pyridinium that has a 10-carbon spacer connecting the rings, a spacing between nitrogen atoms comparable to that of decamethonium. Unfortunately, the efficacy of P107 as a treatment for soman intoxication has not been published, so it is not possible to compare binding strength with effectiveness. However, it should be noted that even P107 binds very weakly compared to typical muscarinic antagonists such as atropine and scopolamine (see Table 1). The binding of 2-PAM apparently has nothing to do with the oxime function because 2-methylpyridine displaced the radioactive ligand at the same concentration as 2-PAM, as shown in Table 1. Neither compound bound very strongly.

Nicotinic binding. Only ten of the thirty-two compounds tested showed any measurable effect on BGT binding to the nicotinic receptors. Again with one exception, all of the effective compounds belong to the series of "P" compounds. The results of the binding experiments are shown in Table 2. The compounds that were ineffective (NB) or marginally effective (>800 μM IC_{50}) include some that have been shown to be therapeutically beneficial against soman poisoning [7], i.e. SAD-128, HY-10, HH-38, HH-39, HH-54, and HS-6. Ketamine, mecamylamine, mikedimide, stelazine, tiletamine, and xylocaine were included at the suggestion of Mr. Walter Sultan, of this Institute, who found that they also have protective effects against soman*; however, none of them affected the binding of BGT at 800 μM.

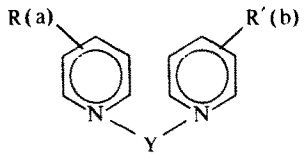
Some of the phosphinates have been reported to protect animals against soman intoxication [19,†]. The following 4-nitrophenyl phosphinates were tested: diphenyl, 4-chlorophenyl(methyl), methyl(phenyl), 4-methoxyphenyl(methyl), dimethyl and di-*n*-butyl. None of them was effective at inhibiting BGT binding to the nicotinic receptors.

The concentration of competing ligand that reduces the binding rate by 50% is defined as the IC_{50}. This parameter is proportional to (but not necessarily equal to) the K_D of the competing ligand [15]. The IC_{50} values obtained for those compounds that were bound by the nicotinic receptors are included in Table 2. The values determined by our procedure for decamethonium and *d*-tubocurarine are also included as references for comparison. Those compounds that bound are good nicotinic ligands, with IC_{50} values ranging from 7 to 61 μM. This compares favorably with *d*-tubocurarine, which has an IC_{50} of

* W. E. Sultan, personal communication, cited with permission.

† C. N. Lieske, J. H. Clark, H. G. Meyer, J. R. Lowe, M. A. Lawson, W. J. Lennox, W. E. Sultan, A. Kaminskis, W. A. Groff, M. B. Shutz and A. Singer, Abstr. No. MEDI 71. Presented at the 181st National Meeting of the American Chemical Society, Atlanta, GA (1981).

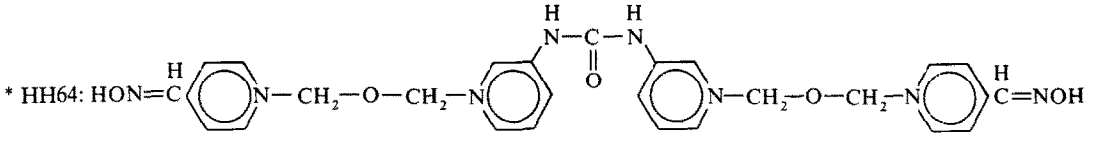
Table 1. Muscarinic receptors: Structures and relative binding strengths of the bis-quaternary salts and other effectors tested



Effector	R	Ring position a	R'	Ring position b	Y	IC_{50} (μM)
TMB-4	—HC=NOH	4	—HC=NOH	4	$-(CH_2)_3$	280
SAD-128	$-C(CH_3)_3$	4	$-C(CH_3)_3$	4	$-CH_2-O-CH_2-$	100
P61	$-C(CH_3)_3$	4	$-C(CH_3)_3$	4	$-(CH_2)_2-$	241
P62	$-C(CH_3)_3$	4	$-C(CH_3)_3$	4	$-(CH_2)_3-$	160
P63	$-C(CH_3)_3$	4	$-C(CH_3)_3$	4	$-(CH_2)_4-$	176
P64	$-C(CH_3)_3$	4	$-C(CH_3)_3$	4	$-(CH_2)_5-$	61.5
P65	$-C(CH_3)_3$	4	$-C(CH_3)_3$	4	$-(CH_2)_6-$	36.6
P66	$-C(CH_3)_3$	4	$-C(CH_3)_3$	4	$-(CH_2)_7-$	32.7
P105	$-C(CH_3)_3$	4	$-C(CH_3)_3$	4	$-(CH_2)_8-$	37.0
P106	$-C(CH_3)_3$	4	$-C(CH_3)_3$	4	$-(CH_2)_9-$	11.4
P107	$-C(CH_3)_3$	4	$-C(CH_3)_3-$	4	$-(CH_2)_{10}-$	6.7
HH-64*						159
HY-10	$-HC=NOC(CH_3)_3$	4	$-HC=NOC(CH_3)_3$	4	$-CH_2-O-CH_2$	800
2-PAM	—CH=NOH	2			CH_3	500

Other compounds

Effector	IC_{50} (μM)
Scopolamine	0.007
Atropine	0.018
Benactyzine	0.40
Procaine	500
2-Methylpyridine	500



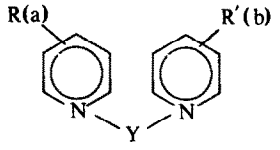
The relative binding strengths are expressed as IC_{50}, the concentration of test compound that displaces 50% of specifically bound [^{3}H]QNB from rat brain synaptic membranes under equilibrium conditions. [^{3}H]QNB concn = 6.9 nM.

6 μM. The therapeutic ED_{50} values of these compounds span a range of two orders of magnitude (see Table 3), with compound P65 being most efficacious. The range of nicotinic binding strengths appears to be at least as great, but with a more noticeable all-or-none characteristic. Among the "P" compounds, those that were most effective in the treatment of soman were also the most strongly bound. Unfortunately, there are no published efficacy data for those compounds that are the strongest anti-nicotinics.

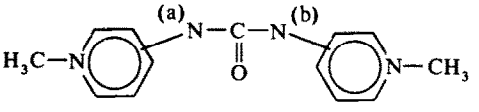
DISCUSSION

In general, it would appear that those compounds that inhibited binding of the nicotinic antagonist BGT are therapeutically effective against soman. Those that also displaced QNB from muscarinic receptors were more effective than those that have no muscarinic activity. However, not all of the effective compounds were able to inhibit BGT or QNB binding. In particular, HH-38, HH-54, and HY-10 showed very little effect on either nicotinic or muscarinic binding, yet all of them are quite effective in protecting against soman [7]. Thus, one is forced to the conclusion that the "P" compounds are unique in their ability to protect against soman intoxication by virtue of their binding to acetylcholine receptors, whereas other compounds with similar structures (such as HH-54 or HY-10) operate by a different mechanism, or else the anticholinergic properties of these compounds have little to do with their therapeutic effectiveness. Kuhnen-Clausen *et al.* [8] were

Table 2. Nicotinic receptors: Structures and relative binding strengths of the bis-quaternary salts and other effectors tested



Effector	R	Ring position a	R'	Ring position b	Y	IC_{50} (μM)
Toxogonin	—HC=NOH	4	—HC=NOH	4	—CH_2—O—CH_2—	NB*
3,3'-LuH-6	—HC=NOH	3	—HC=NOH	3	—CH_2—O_1—CH_2—	>800
TMB-4	—HC=NOH	4	—HC=NOH	4	—$(CH_2)_3$—	NB
HS-6	—HC=NOH	2	—$CONH_2$	3	—CH_2—O—CH_2	NB
HY-10	—HC=NOC$(CH_3)_3$	4	—HC=NOC$(CH_3)_3$	4	—CH_2—O—CH_2	>800
HY-18	—C$(CH_3)_3$	4	—HC=NOH	2	—CH_2—O—CH_2	>800
SAD-128	—C$(CH_3)_3$	4	—C$(CH_3)_3$	4	—CH_2—O—CH_2	>800
P61	—C$(CH_3)_3$	4	—C$(CH_3)_3$	4	—$(CH_2)_2$—	61
P62	—C$(CH_3)_3$	4	—C$(CH_3)_3$	4	—$(CH_2)_3$—	45
P63	—C$(CH_3)_3$	4	—C$(CH_3)_3$	4	—$(CH_2)_4$—	20
P64	—C$(CH_3)_3$	4	—C$(CH_3)_3$	4	—$(CH_2)_5$—	12
P65	—C$(CH_3)_3$	4	—C$(CH_3)_3$	4	—$(CH_2)_6$—	12
P66	—C$(CH_3)_3$	4	—C$(CH_3)_3$	4	—$(CH_2)_7$—	20
P105	—C$(CH_3)_3$	4	—C$(CH_3)_3$	4	—$(CH_2)_8$—	30
P106	—C$(CH_3)_3$	4	—C$(CH_3)_3$	4	—$(CH_2)_9$—	7
P107	—C$(CH_3)_3$	4	—C$(CH_3)_3$	4	—$(CH_2)_{10}$—	14



Effector	a	b	IC_{50} (μM)
HH-38	3	3	>800
HH-39	4	4	>800
HH-54	3	4	>800

Other compounds

Effector	IC_{50}* (μM)
Edrophonium bromide (Tensilon)	15
d-Tubocurarine	6
Decamethonium bromide	25
Procaine	>800

* IC_{50} is the concentration of test compound that reduces the rate of binding of α-bungarotoxin to 50% of its normal rate.

† NB, no measurable effect on α-bungarotoxin binding at 8×10^{-4} M.

also unable to demonstrate a correlation between therapeutic efficacy against soman and the antagonism of nicotinic binding in a series of bis-quaternary oximes. However, both Kuhnen-Clausen and Amitai *et al.* [13] found a positive correlation between muscarinic binding activity and therapeutic efficacy. It should be pointed out that the compounds used by Kuhnen–Clausen and most of those used by Amitai are oximes and thus pose the possibility that their therapeutic effectiveness may have been due, at least in part, to reactivation of AChE. Most of the compounds tested here have no oxime group, so they cannot be acting as AChE reactivators. It should also be pointed out that the experiments reported here were done under conditions in which the binding sites were saturated, using very high concentrations of radiolabeled ligand. This decreases the sensitivity of the test because a concomitantly high concentration of the test compound must be used to displace it. However, we felt that these conditions would reduce the possibility that some sites, particularly those of low affinity for QNB, would be missed. Since QNB binds to all of the muscarinic receptor subtypes, it is expected that this procedure would detect any type of muscarinic binding sites. However, we have not yet determined whether those

Table 3. Relationship between the binding of bis-quaternary salts to acetylcholine receptors and their effectiveness in the treatment of soman poisoning

Compound	IC_{50} (musc) (μM)	IC_{50} (nic) (μM)	ED_{50}* (moles/kg $\times 10^5$)
P65	36.6	12	0.013
P66	32.7	20	0.13
HH-54	NB†	>800	0.38
HY-10	800	>800	0.56
P64	61.5	12	0.58
P63	174	20	0.62
HH-39	>1000	>800	1.02
HH-38	>1000	>800	1.04
P62	160	45	1.76
P61	>1000	61	2.01
SAD-128	100	>800	2.15
Toxogonin	42‡	NB	NE§

* Compounds are listed in order of their reported efficacy [7] in the treatment of soman intoxication. The ED_{50} of the tested compound reduces the toxic effect of an LD_{95} of soman to a level of 50% mortality. While P105, P106 and P107 bound strongly at both nicotinic and muscarinic receptors, there are no published efficacy data available.

† NB, no measurable effect on radioligand binding at 8×10^{-4} M.

‡ Value was determined by Amitai *et al.* [13].

§ NE = not effective.

compounds that displace QNB are subtype specific.

Table 3 lists the binding data for those compounds that have been listed for efficacy as a therapy against soman [7]. The compounds are listed in order of their therapeutic efficacy. It is apparent from inspection of this table that neither nicotinic nor muscarinic binding correlates well with efficacy. Thus, it would appear that the mechanism of protection does not involve the acetylcholine receptors measured by either BGT or QNB binding.

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