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IN VIVO PROTECTION AGAINST SOMAN TOXICITY BY KNOWN INHIBITORS OF ACETYLCHOLINE SYNTHESIS *IN VITRO*

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Abstract—Soman inhibits the enzyme acetylcholinesterase, essentially irreversibly, producing an accumulation of acetylcholine (ACh) which is responsible for many of its toxic effects. Current approaches to treatment include: (1) atropine, a muscarinic receptor blocker; (2) pyridine-2-aldoxime methylchloride (2-PAM), an enzyme reactivator; and (3) carbamate protection of the enzyme. However, no fully satisfactory regimen has been found, primarily because of the rapid aging process. In this study, compounds known to inhibit ACh synthesis *in vitro* were evaluated in combination with atropine and 2-PAM so as to assess their potential utility in protection against soman toxicity in rats. Acetyl-secohemicholinium (100 μg/kg, i.c.v.t., 30 min prior to soman), an inhibitor of high affinity choline uptake (HAChU) and cholineacetyltransferase (ChAT) activity *in vitro*, enhanced the protective effects of atropine and 2-PAM, reducing the mortality within the first 2 hr following soman. *N*-Hydroxyethylnaphthylvinylpyridine (NHENVP), a quaternary ChAT inhibitor (1.7 μmol/kg, i.m.), significantly reduced the overall percent mortality due to soman from 80% to 20%. The compound was most effective when administered 2–3 min prior to soman and was effective only by the intramuscular route. *N*-Allyl-3-quinuclidinol, a potent HAChU inhibitor (1 μmol/kg, i.m.) was the most effective quinuclidine analog evaluated, also reducing the percent mortality for a 24-hr period. Unlike NHENVP, it was most effective when given 30–60 min prior to soman. It is suggested from the data that compounds that disrupt presynaptic ACh synthesis *in vitro* may prove effective in treating organophosphate poisoning. The results demonstrate interesting differences among the compounds studied and provide insight for the design of protectants against soman toxicity. These findings further underscore the need to examine the structure activity and pharmacokinetic properties of these compounds, i.e. comparison of routes of administration, dose–response relationships, and time to effect.

Soman (1,2,2-trimethyl-propyl-methylphosphonofluoridate; pinacolyl-methylphosphonofluoridate) is a highly toxic organophosphorous compound. It acts by phosphonylating the serine hydroxyl at the active site of acetylcholinesterase (acetylcholine acetylhydrolase; AChE; EC 3.1.1.7), producing essentially irreversible inhibition. Thus, many of its toxic effects are due to the rapid accumulation of acetylcholine (ACh) [1]; these include peripheral muscarinic manifestations such as excessive secretions and smooth muscle contractions, exaggerated nicotinic responses characterized by skeletal muscle fasciculations and weakness, and central effects manifested as seizures, all leading to death by respiratory depression and cardiovascular collapse.

Attempts to treat organophosphorous poisoning have concentrated on two approaches: (a) post-exposure administration of atropine, a muscarinic receptor antagonist in combination with artificial respiration and enzyme reactivation by oximes [2–4] and (b) pre-exposure carbamate protection of the enzyme [5–9]. Readers are referred to a recent review of these antidotes for a more complete discussion [10]. To date, these therapeutic approaches have only met with partial success in reversing soman toxicity, mostly due to the rapid "aging" process of the phosphonylated enzyme, in which the enzyme is rendered insensitive to reactivation by oximes. Addition of mecamylamine, a centrally active nicotinic antagonist and ganglionic blocker, to atropine and oxime or carbamate, has been found to provide increased protection against organophosphorous poisoning in animals [9, 11], suggesting that alternative inhibition of the cholinergic system is of benefit in treatment of soman toxicity.

Since the acute toxicity of soman is due to excessive accumulation of ACh, this study sought to examine whether compounds known to suppress presynaptic synthesis of ACh *in vitro* would enhance the protective regimen *in vivo*. To date, a limited number of studies using compounds of this type have been

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reported. Schoene *et al.* [12] and Harris *et al.* [13] found that naphthylvinylpyridinium, a choline acetyltransferase (ChAT) inhibitor, reduces soman toxicity in mice. Harris *et al.* [13] also demonstrated that hemicholinium-3, a potent inhibitor of high affinity choline uptake (HAChU), administered intracerebroventricularly (i.c.v.t.) protects against soman intoxication. Hopff *et al.* [14] found that methyl methane thiol sulfonate, another ChAT inhibitor, prolongs the time to death of a lethal dose of both sarin and soman in mice. It was also reported that clonidine, an alpha-2 adrenergic receptor agonist, known to inhibit ACh release, protects against anticholinesterase toxicity in mice [15].

The current work involved the design, synthesis and testing of quinuclidine inhibitors of ChAT activity and HAChU as potential protectants. The abilities of these compounds to protect against soman were compared to hemicholinium and naphthylvinylpyridine derivatives. Preliminary results were reported previously [16, 17].

MATERIALS AND METHODS

Chemicals. Soman (pinacolyl methylphosphonofluoridate) was obtained from the U.S. Army Institute for Chemical Defense. Atropine sulfate and pyridine-2-aldoxime methylchloride (2-PAM) were purchased from the Sigma Chemical Co. (St. Louis, MO).

Mecamylamine was obtained from Merck, Sharp & Dohme (West Point, PA) and pempidine was purchased from Aldrich Chemicals (Milwaukee, WI). *N*-Hydroxyethyl-naphthylvinylpyridine was obtained from Calbiochem (San Diego, CA). Acetylsecohemicholinium, naphthylvinylpyridine and quinuclidine derivatives were synthesized in these laboratories.

General procedures. Male Sprague–Dawley rats (West Jersey Biological, Wenonah, NJ), 100–125 g, were used in all experiments. The animals were housed in a controlled environment facility with a 12 hr–12 hr light–dark cycle and were given laboratory chow and water, *ad lib.* The LD_{50} for soman was determined periodically using probit analysis, and the dose was checked on the morning of each experiment. The protocol of a typical daily study was as follows:

Group I: Rats received soman (128–130 μg/kg, approximately LD_{80}), subcutaneously at time zero.

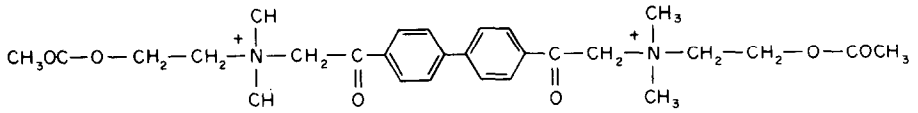
Group II: Rats received soman (as in Group I) followed 1 min later by intramuscular administration of atropine sulfate (16 mg/kg) and 2-PAM (30 mg/kg).

Group III, etc.: Rats received soman, atropine and 2-PAM (as above). Prior to soman, animals were treated with one of the compounds to be evaluated, administered intramuscularly or intracerebroventricularly between 2 and 120 min prior to soman exposure, as noted. Structures of selected compounds are shown in Fig. 1.

Each test group contained ten animals.

Following soman, animals were observed for 24 hr, during which behavioral manifestations and survival time were reported. The dose chosen for each of the compounds to be evaluated was based on their K_i value for inhibition of choline acetyltransferase activity or high affinity choline uptake determined by *in vitro* assay [18–20]. Each of the compounds was given as a single dose.

Statistical analysis. Significant differences in the percent mortality among drug treatments were determined by paired *t*-tests [21].



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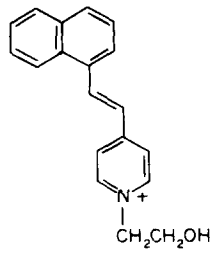

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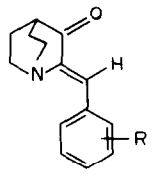

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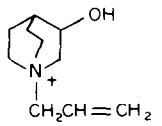

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Fig. 1. Structures of selected inhibitors of acetylcholine synthesis, *in vitro*. Key: (I) acetylsecohemicholinium (AcSecoHC); (II) *N*-hydroxyethylnaphthylvinylpyridine (NHENVP); (III) 2-benzylidene-3-quinuclidinone (2-B-3-Q); and (IV) *N*-allyl-3-quinuclidinol (NAQ).

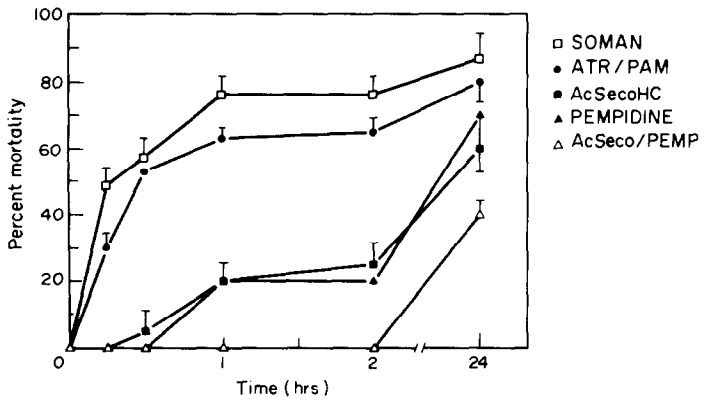

Fig. 2. Protection against soman toxicity by acetylsecohemicholinium (AcSecoHC, 100 μg/kg, i.c.v.t. 30 min prior to soman), pempidine (2.5 mg/kg, i.m., 2 min prior to soman), or both when given in conjunction with atropine sulfate (16 mg/kg) and 2-PAM (30 mg/kg) at 1 min post-soman. The dose of soman given to all animals was 128 μg/kg subcutaneously at time zero. Each point represents the mean ± SEM of three to six experiments.

RESULTS

The LD_{50} of soman following subcutaneous administration in rats was 123.4 ± 5.5 μg/kg at 1 hr and 93.5 ± 4.3 μg/kg at 24 hr. At doses between 128 and 130 μg/kg, those used in our studies, the cumulative percent mortality from soman, which includes data from several experiments, was approximately 68% within 1 hr and 80% in 24 hr. Administration of atropine sulfate and 2-PAM at 1 min post-soman reduced the overall percent mortality to 51.6% in 1 hr and 61.6% in 24 hr. A reduction in mortality was not uniformly observed in all experiments.

To determine whether known inhibitors of ACh synthesis would enhance protection against soman toxicity, acetylsecohemicholinium (AcSecoHC, 100 μg/kg, i.c.v.t.), an inhibitor of HAChU and ChAT activity *in vitro*, was administered 30 min prior to soman (Fig. 2). The compound reduced markedly the percent mortality within the first 2 hr when given in conjunction with atropine/2-PAM. Though AcSecoHC failed to reduce overall mortality at 24 hr, it delayed markedly the time to death.

Given that AcSecoHC is a bisquaternary ammonium compound also possessing a lipophilic biphenyl moiety, it is conceivable that it possesses activities in addition to the inhibition of ACh synthesis; one of those may be alteration of nicotinic activity. This led to the testing of pempidine and mecamylamine, non-quaternary nicotinic antagonists and ganglionic blockers. Pempidine (2.5 mg/kg, i.m. at 2 min prior to soman) reduced the percent mortality within the first 2 hr following soman (Fig. 2). When given in combination with AcSecoHC, atropine and 2-PAM, no deaths occurred in 2 hr, and overall mortality was reduced. Mecamylamine produced similar results.

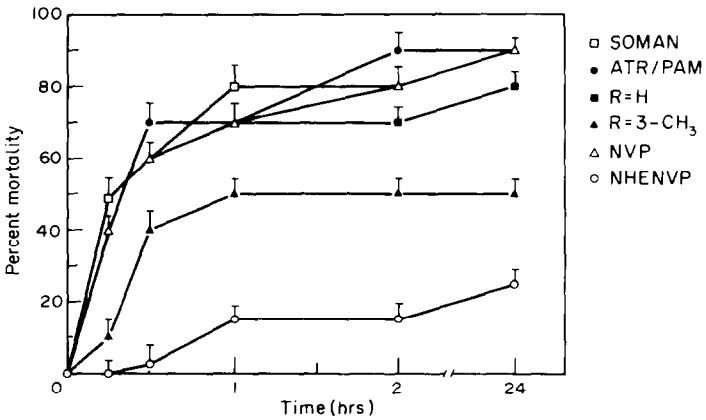

Fig. 3. Protection against soman toxicity by 2-benzylidene-3-quinuclidinone (BQ) and naphthylvinylpyridine (NVP) derivatives. The BQs were given i.m., 30 min prior to soman at a dose of 1 μmol/kg. Atropine sulfate (16 mg/kg) and 2-PAM (30 mg/kg) were given i.m. at 1 min post-soman. Derivatives shown are 2-benzylidene-3-quinuclidinone (R = H) and 2-(3-methyl)benzylidene-3-quinuclidinone (R = 3—CH_3). NVP and *N*-hydroxyethyl NVP (NHENVP) were given at 1.7 μmol/kg, i.m., at 30 min prior to soman.

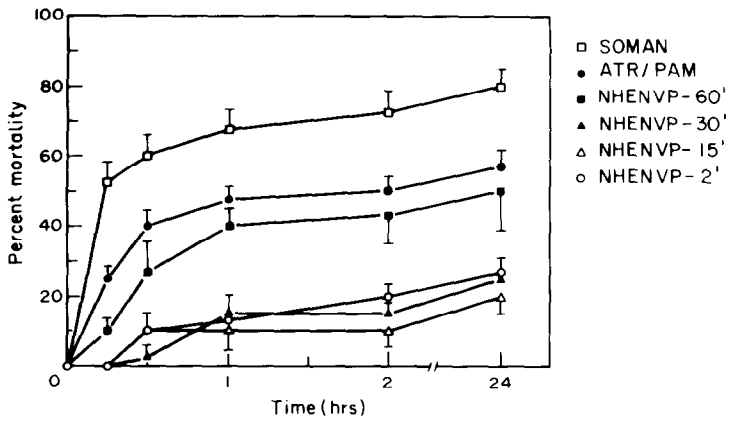

Fig. 4. Protection against soman toxicity by *N*-hydroxyethylnaphthylvinylpyridine. NHENVP was administered intramuscularly at various times (2–60 min) prior to soman. The dose of inhibitor chosen was 1.7 μM, approximately the K_i for inhibition of ChAT activity, *in vitro*. Animals were given soman followed by atropine/2-PAM as described in Materials and Methods. Each point represents the mean ± SEM of three to six experiments.

In a second series of experiments, the effectiveness of several ChAT inhibitors was assessed (Fig. 3). *N*-Hydroxyethylnaphthylvinylpyridine (NHENVP, 1.7 μmol/kg, i.m. at 30 min prior to soman, see Fig. 1 for structure) was the most effective in protecting against soman, reducing overall mortality f om 80% to 20% at 24 hr. The compound was ineffective when administered i.c.v.t. at the same dose, and was most protective when given 2–30 min prior to soman; its effectiveness decreased when given 1 hr prior to soman (Fig. 4). The non-quaternary homolog, naphthylvinylpyridine, failed to reduce soman toxicity at concentrations up to ten times that of the quaternary compound.

The 2-benzylidene-3-quinuclidinones developed in these laboratories are noncompetitive, nonquaternary inhibitors of ChAt activity *in vitro* [19]. The only effective derivative with regard to *in vivo* protection was 2-(3-methyl)benzylidene-3-quinuclidinone which slightly reduced overall mortality. The compound was most effective when given 30 min prior to soman by the i.m. route, yet more effective i.c.v.t. when given 2 min prior to soman. Other benzylidene quinuclidinone derivatives evaluated but failing to demonstrate protection by either i.c.v.t. or i.m. administration were the unsubstituted, 3-chloro and 3,4-dichloro analogs.

Several quinuclidinyl inhibitors of HAChU were evaluated for protection against soman toxicity (Fig. 5). Given intramuscularly at 1 μmol/kg 30 min prior to soman, *N*-allyl-3-quinuclidinol (NAQ) dramatically reduced soman toxicity from an LD_{70} to an LD_{20} at 1 hr and from LD_{76} to LD_{35} at 24 hr post-soman. The corresponding ketone, *N*-allyl-3-quinuclidinone, had no effect, whereas *N*-methyl-3-quinuclidinone reduced toxicity to an LD_{30} at 1 hr. Especially noteworthy was the observation that, behaviorally, these animals appeared somewhat sedated; they did

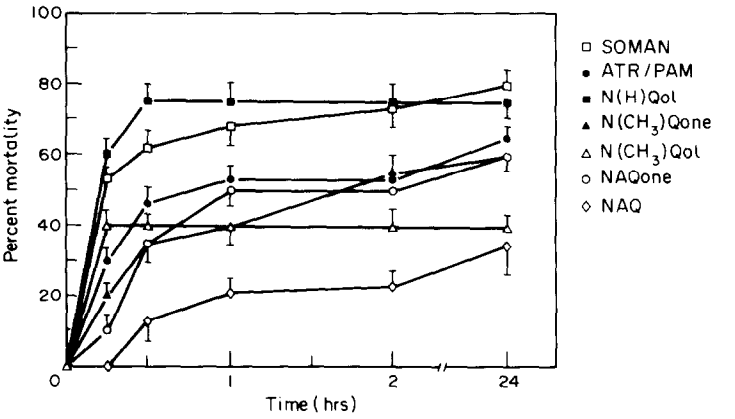

Fig. 5. Protection against soman toxicity by quinuclidinyl inhibitors of HAChU. All compounds were evaluated in conjunction with atropine/2-PAM at a dose of 1 μmol/kg given intramuscularly at 30 min prior to soman. The derivatives evaluated were 3-quinuclidinol hydrochloride (N(H)Qol), *N*-methyl-3-quinuclidinone ($N(CH_3)$Qone), *N*-methyl-3-quinuclidinol ($N(CH_3)$Qol), *N*-allyl-3-quinuclidinone (NAQone) and *N*-allyl-3-quinuclidinol (NAQ). Each point represents the mean ± SEM of three to six experiments.

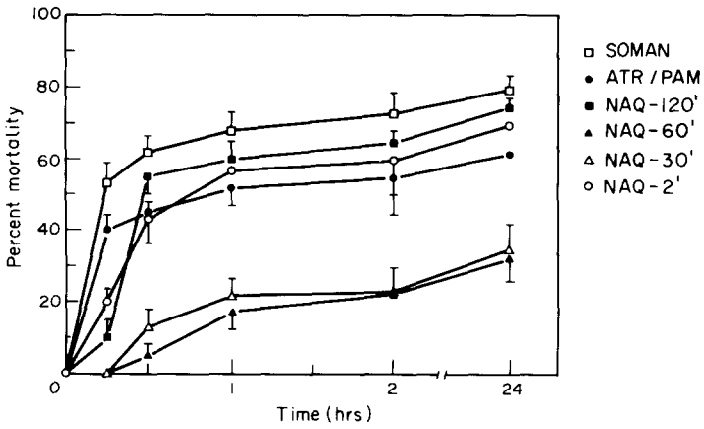

Fig. 6. Protection against soman toxicity by *N*-allyl-3-quinuclidinol (NAQ). The *N*-allyl derivative was administered intramuscularly at various times (2–120 min) prior to soman. The dose of inhibitor chosen was 1 μmol/kg, approximately the K_i for inhibition of HAChU, *in vitro*. All animals were given 128 μg/kg soman at time zero followed by atropine and 2-PAM at 1 min. Each point represents the mean ± SEM of three to six experiments.

not exhibit the tremors, profuse salivation, and gagging characteristically observed with this dose of soman. Since these compounds contain a quaternary nitrogen, only peripheral activity would be expected when given i.m. In contrast, when given i.c.v.t. at 1 μmol/kg, 30 min prior to soman, the compounds were ineffective. The tertiary amine, 3-quinuclidinol hydrochloride, a derivative which failed to inhibit HAChU or ChAT activity *in vitro*, did not protect against soman. In examining the time-to-effect (Fig. 6), it should be noted that, in contrast to NHENVP, NAQ was most effective when given 30–60 min prior to soman.

DISCUSSION

We have begun to examine presynaptic events in order to determine whether inhibition of ACh synthesis may enhance protection against soman toxicity. Several compounds, which *in vitro* inhibit ACh synthesis, were evaluated *in vivo* in conjunction with atropine/2-PAM as potential protectants in the rat. AcSecoHC (i.c.v.t.) reduced the mortality within the first 2 hr following soman. The protection afforded by this route established the principle that an inhibitor of HAChU with access to the central nervous system may be of utility in preventing the toxicity of organophosphates. The failure of AcSecoHC to reduce overall mortality at 24 hr may have been due to the fact that only a single dose of the compound was administered; multiple dosing might lead to a more lasting protection. Pempidine, a nonquaternary nicotinic blocker, added to the protection of AcSecoHC, confirming the work of Harris *et al.* [11] and Heyl *et al.* [9] which demonstrated the protective effects of the related compound mecamylamine.

However, though the aforementioned compounds had access to the CNS, NAQ and NHENVP, both quaternary compounds, were only effective when administered intramuscularly. This brings up a dilemma as to the relative importance of peripheral versus central events as contributors to soman toxicity. It is possible that, despite the route of administration, the protective effects of AcSecoHC, NAQ and NHENVP are through a peripheral mechanism, since compounds such as AcSecoHC administered i.c.v.t. may distribute into the periphery. It is also possible that, in soman poisoning, a change in blood–brain barrier may permit NAQ or NHENVP to enter the CNS. The fact that these quaternary compounds were ineffective following central administration may have been related to the dose or time course chosen. Shih [22] has demonstrated rapid increases in ACh levels in multiple brain regions following soman exposure. Preliminary studies in our laboratories have demonstrated that pretreatment of animals with AcSecoHC prevented soman-induced increases in cortex and striatum, whereas NAQ prevented increases only in striatal ACh. This suggests that central inhibition of ACh synthesis may be effective against soman toxicity [23]. Further studies are underway to elucidate the mechanism of these agents.

Of the quinuclidines tested, compounds which inhibit HAChU *in vitro* appeared to be markedly more effective than inhibitors of ChAT activity as potential protectants; *N*-allyl-3-quinuclidinol, a potent inhibitor of HAChU (K_i = 1–6 μM [18]), was the most effective. Another quinuclidine derivative, 3-quinuclidinol hydrochloride, which failed to inhibit either ChAT or HAChU *in vitro* [18], was ineffective in reversing the toxicity of soman. However, some quinuclidines, which do inhibitor HAChU [18], also failed to protect against soman at the doses given. We must therefore ask if NAQ has another pharmacologically important attribute determining its protective effect.

In contrast to the quinuclidines, the ChAT inhibitor NHENVP provided substantial protection against soman. This confirms previous work showing protection by ChAT inhibitors [12, 14]. However, in our work, the nonquaternary derivative, NVP, failed to protect even at ten times the concentration. This

may be significant since NHENVP ($K_i = 1.7\ \mu M$) is approximately ten times more potent as an inhibitor of ChAT [19].

The rat is a very difficult animal in which to show protection; thus, the protection achieved in this study is worthy of note. The most significant outcome of the present study is that compounds which *in vitro* interfere with the formation of ACh provide *in vivo* protection against soman when combined with atropine and 2-PAM. There is, however, no direct correlation, with few exceptions, between potency for inhibition of HAChU or ChAT activity and protective efficacy. The difference in time-interval for optimal dosing found between NAQ and NHENVP may be a function of pharmacologic mechanism and/or pharmacokinetic properties.

Studies are underway to determine the relative levels of brain ACh in soman-intoxicated rats following pretreatment with these compounds, in an attempt to correlate these levels with protective effects. Nevertheless, even lacking this information, these findings indicate that moieties known to inhibit ACh synthesis represent structural classes that may lead to the development of better treatment for soman poisoning.

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