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HI-6: REACTIVATION OF CENTRAL AND PERIPHERAL ACETYLCHOLINESTERASE FOLLOWING INHIBITION BY SOMAN, SARIN AND TABUN *IN VIVO* IN THE RAT*

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Abstract—HI-6, ([[[(4-aminocarbonyl)pyridino]methoxy]methyl]-2-[(hydroxyimino)methyl]-pyridinium dichloride), is an oxime which, when combined with atropine, is an extremely effective therapy against organophosphate poisoning. It was found that, following soman (287 μg/kg) poisoning, HI-6 reactivated acetylcholinesterase in the diaphragm and intercostal muscles but not in the brain. At a lower dose of soman (110 μg/kg), HI-6 reactivated acetylcholinesterase in the brain. HI-6 reactivated sarin-inhibited acetylcholinesterase in the brain and in the respiratory musculature but did not reactivate tabun-inhibited acetylcholinesterase. It was also found that soman produced a differential inhibition of diaphragm and intercostal muscle acetylcholinesterase *in vivo*, whereas the *in vitro* I_{50} for soman was the same in both areas. HI-6 was capable of reactivating soman-inhibited acetylcholinesterase when administered up to 30 min post-soman, indicating that the rate of aging of the soman–acetylcholinesterase complex is slower than previously reported. The above results suggest that, in severe soman poisoning, the primary lesion occurs in peripheral acetylcholinesterase in the respiratory musculature (specifically the diaphragm).

Soman (methylphosphonofluoridic acid 1,2,2-trimethylpropylester) poisoning is resistant to the conventional therapy employing atropine plus an oxime. However, in recent years some new bispyridinium oximes, originating from the laboratory of Professor Hagedorn in Freiburg, Germany, when combined with atropine are extremely effective against soman poisoning in mice [1–3], rats [2, 4, 5], dogs [6, 7] and monkeys [8]. HI-6, [[[(4-aminocarbonyl)-pyridino]methoxy]methyl] - 2 - [(hydroxyimino)-methyl]-pyridinium dichloride (Fig. 1), was found to be the least toxic and most efficacious oxime against soman poisoning in mice [1].

The purpose of this study was to investigate the mechanism of the therapeutic action of HI-6 with respect to its ability to reactivate organophosphate-inhibited acetylcholinesterase in various areas of the central and peripheral nervous systems.

MATERIALS AND METHODS

Male Sprague–Dawley rats (200–250 g) were used in this study. The organophosphates were injected subcutaneously (s.c.) 30 min after an intraperitoneal (i.p.) injection of atropine sulfate. HI-6 was administered i.p. immediately following the organophosphate except in the rate of aging experiments where the HI-6 administration was delayed for various times.

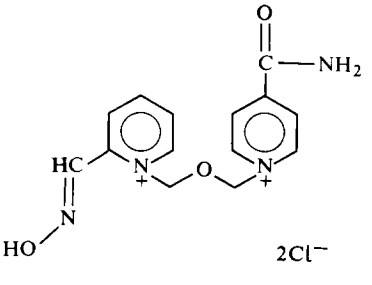

Fig. 1. Structure of HI-6.

Acetylcholinesterase determination. Rats were decapitated and exsanguinated. The brain was removed and rinsed in cold saline. Samples of the pons-medulla, cortex and striatum were blotted dry, weighed, and homogenized in a 0.1 M phosphate buffer (pH 7.4) and 0.4 M sucrose. The homogenate was diluted to give a 1% homogenate (w/v).

From these same animals the entire diaphragm minus the central clear tendinous portion, and a sample of the intercostal muscle were rinsed in saline, blotted dry, and weighed. These tissues were then frozen in liquid nitrogen and pulverized. The frozen powdered tissue was then homogenized for 1 min in the cold in phosphate–sucrose buffer using an Ultra-Turrax homogenizer at a setting of 30. The homogenate was diluted to give a 10% solution (w/v).

The acetylcholinesterase activity was determined by the procedure of Siakotos *et al.* [9] utilizing [^{14}C]acetylcholine iodide as the substrate. The time of incubation for cortex, pons-medulla, diaphragm and intercostal muscle was 10 min, whereas the time of incubation for the striatum was 5 min.

In experiments to determine the rate of aging of soman-inhibited acetylcholinesterase *in vivo*, each animal was administered atropine sulfate (17.4 mg/kg, i.p.) 30 min prior to receiving soman (110 μg/kg, s.c.) at zero time. At various times after receiving soman the animals were administered HI-6 (125 mg/kg, i.p.). All animals were killed 3 hr after receiving soman, and the diaphragm and intercostal muscles were prepared as described above.

Statistical significance was determined using Student's unpaired *t*-test.

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Materials. Soman, sarin (methylphosphonofluoridic acid 1-methylethylester), tabun (dimethylphosphoramidocyanidic acid, ethylester) and all the bispyridinium compounds used in this study were synthesized by the Organic Chemistry Section, Defence Research Establishment Suffield. The organophosphates were 96–98% pure and the bispyridinium compounds were >98% pure. Aqueous solutions of all the compounds were prepared immediately prior to injection and diluted to the required concentration with 0.9% NaCl.

RESULTS

The results in Table 1 demonstrate that, in the rats which received only atropine (37.5 mg/kg, i.p.) 30 min before soman (287 μg/kg, s.c.) and died (all animals receiving atropine + soman died within 10 min), the acetylcholinesterase activity was decreased in all areas examined. The acetylcholinesterase activity in the respiratory musculature was the most severely affected, followed by pons-medulla, striatum and cortex, in that order. Rats receiving atropine, soman, and HI-6 (125 mg/kg) and surviving for various periods of time were killed, and the acetylcholinesterase activity was determined. In the brain regions of rats that had received HI-6 therapy and survived for 30 min after soman, the acetylcholinesterase activity tended to be lower than in those that died following soman and atropine. The difference was significant in the striatum ($P < 0.001$). The acetylcholinesterase levels appeared to be recovering gradually over the 24-hr time period. In the case of the respiratory musculature, significant reactivation of the soman-inhibited acetylcholinesterase had occurred by 30 min and reached a higher level by 60 min. Rats that had died after receiving the treatment of atropine + HI-6 + soman had no detectable acetylcholinesterase activity in the diaphragm, whereas the various brain areas had acetylcholinesterase activity which was not significantly different from that in rats receiving atropine + HI-6 + soman and surviving for 30 min after soman treatment.

The lack of reactivation of soman-inhibited brain acetylcholinesterase suggested that perhaps HI-6, since it is a bisquaternary amine, did not pass the blood–brain barrier. However, the results in Table 2 demonstrate that, in the case of poisoning by sarin, HI-6 (125 mg/kg) produced significant reactivation of acetylcholinesterase activity in the pons-medulla, cortex and striatum and complete reactivation of the acetylcholinesterase present in the diaphragm and intercostal muscles. Thus, HI-6 can reactivate sarin-inhibited brain acetylcholinesterase; however, the results in Table 3 show that HI-6 had no significant effect on tabun-inhibited acetylcholinesterase. Also, SAD-128, the non-oxime bispyridinium compound, was found to protect rats to a limited degree against soman (287 μg/kg). In this case, no reactivation of central or peripheral acetylcholinesterase was evident (J. G. Clement, unpublished observations).

In another series of experiments, the dose of soman was reduced to one LD_{50} (110 μg/kg) and atropine was reduced to 17.4 mg/kg, and the ability of

Table 1. Effect of HI-6 (125 mg/kg) on acetylcholinesterase activity in rats following soman (287 μg/kg) poisoning

Group	Pons-medulla	%	Striatum	%	Cortex	%	Diaphragm	%	Intercostals	%
Control	7.83 ± 0.56*	100	40.00 ± 4.03	100	4.36 ± 0.58	100	0.82 ± 0.12	100	1.05 ± 0.06	100
Atropine + soman†	0.23 ± 0.09	2.9	1.90 ± 0.10	4.7	0.36 ± 0.15	8.3	0.01 ± 0.00	1.2	0.02 ± 0.01	1.9
Atropine + soman + HI-6‡										
0.5 hr	0.13 ± 0.02	1.7	0.74 ± 0.14§	1.8	0.30 ± 0.04	6.9	0.15 ± 0.02§	18.3	0.15 ± 0.05§	14.3
1 hr			2.04 ± 0.08	5.1	0.33 ± 0.05	7.6	0.26 ± 0.05§	31.7	0.46 ± 0.01§	43.8
2 hr	0.29 ± 0.08	3.7	1.94 ± 0.14	4.9	0.25 ± 0.10	5.7	0.26 ± 0.01§	31.7	0.32 ± 0.02§	30.5
24 hr	1.80 ± 0.06§	22.6	4.40 ± 0.32	11.0	0.73 ± 0.16‖	16.7	0.37 ± 0.08§	45.1	0.61 ± 0.18§	58.0
Died following therapy¶	0.17 ± 0.06	2.2	0.64 ± 0.12	1.6	0.07 ± 0.04	1.6	0	0	0.04 ± 0.03	3.8

* Values are nmoles acetylcholine hydrolysed (mg tissue)$^{-1}$ · min^{-1} (mean ± S.D., N = 4).
† These animals died within 10 min following soman administration. They were decapitated and exsanguinated at the cessation of respiration. Atropine (37.5 mg/kg, i.p.) was administered 30 min before soman.
‡ Animals that had received HI-6 therapy immediately following soman were killed at the specified times after soman administration.
§ Significantly different from atropine + soman group, $P < 0.001$.
‖ Significantly different from atropine + soman group, $P < 0.02$.
¶ These animals pretreated with atropine had received HI-6 therapy immediately after soman but they died before the 30-min sampling period (usually within 10–20 min post-soman).

Table 2. Effect of HI-6 (125 mg/kg) on acetylcholinesterase activity in rats following sarin (287 μg/kg) poisoning

Group	Pons-medulla	%	Striatum	%	Cortex	%	Diaphragm	%	Intercostals	%
Control	7.83 ± 0.56*	100	40.04 ± 4.03	100	4.36 ± 0.58	100	0.82 ± 0.12	100.0	1.05 ± 0.06	100
Atropine + sarin†	0.37 ± 0.03	4.7	1.00 ± 0.01	2.5	0.34 ± 0.09	7.8	0.04 ± 0.01	4.9	0.13 ± 0.12	12.4
Atropine + sarin + HI-6‡	2.93 ± 0.18	37.4§	6.70 ± 0.09	16.7§	2.37 ± 0.41	54.4§	0.91 ± 0.03	111.0§	0.99 ± 0.01	94.3§

* Values are nmoles acetylcholine hydrolysed · (mg tissue)$^{-1}$ · min^{-1} (mean ± S.D., N = 4).

† Following sarin administration, these animals died within 10–15 min. The animals were decapitated and exsanguinated at the cessation of respiration. Atropine (37.5 mg/kg, i.p.) was administered 30 min before sarin.

‡ These animals were given atropine (37.5 mg/kg, i.p.) 30 min before sarin. One hour after administration of sarin, s.c. + HI-6, i.p., the animals were decapitated and exsanguinated.

§ Significantly different from atropine + sarin group, $P < 0.001$.

Table 3. Effect of HI-6 (125 mg/kg) on acetylcholinesterase activity in rats following tabun (200 μg/kg) poisoning

Group	Pons-medulla	%	Striatum	%	Cortex	%	Diaphragm	%	Intercostals	%
Control	7.83 ± 0.56*	100	40.04 ± 4.03	100	4.36 ± 0.58	100	0.82 ± 0.12	100	1.05 ± 0.06	100
Atropine + tabun†	2.03 ± 0.98	26	13.18 ± 7.89	33	1.22 ± 0.48	28	0.29 ± 0.19	35	0.76 ± 0.27	72
Atropine + tabun + HI-6‡	1.98 ± 0.59	25§	14.34 ± 4.11	36§	1.39 ± 0.50	32§	0.49 ± 0.22	60§	0.82 ± 0.17	78§

* Values are nmoles acetylcholine hydrolysed · (mg tissue)$^{-1}$ · min^{-1} (mean ± S.D., N = 4).

† Animals were injected i.p. with atropine (37.5 mg/kg, i.p.) 30 min before receiving tabun, s.c. (200 μg/kg; ≈ one LD_{50}). The animals were killed 1 hr post-tabun by decapitation and exsanguination.

‡ Same as above except the animals received HI-6, i.p., immediately after s.c. administration of tabun.

§ Not significantly different from atropine + tabun group.

Table 4. Effect of HI-6 (125 mg/kg) on acetylcholinesterase activity in various areas of rat brain following soman (110 μg/kg) poisoning*

Group	Acetylcholinesterase activity					
	Pons-medulla	%†	Cortex	%	Striatum	%
Control	7.83 ± 0.56‡	100	4.36 ± 0.58	100	40.04 ± 4.03	100
Soman + atropine	0.54 ± 0.58	7	0.30 ± 0.33	7	8.01 ± 4.89	20
Soman + atropine + HI-6	2.60 ± 1.23§	33	0.59 ± 0.18‖	13	29.96 ± 5.98	75§

* Animals received soman (110 μg/kg; s.c.) 30 min after atropine (17.4 mg/kg; i.p.). HI-6 (125 mg/kg) was administered i.p. immediately following soman administration. Animals were decapitated 3 hr after receiving the soman.

† Percentage of control acetylcholinesterase activity.

‡ Values are nmoles acetylcholine hydrolysed · (mg tissue)$^{-1}$ · min^{-1} (mean ± S.D., N = 8).

§ Significantly different from soman + atropine group, $P < 0.001$.

‖ Not significantly different from soman + atropine group, $P < 0.05$.

HI-6 (125 mg/kg) to reactivate the soman-inhibited acetylcholinesterase was examined. The results in Table 4 show that, at the reduced doses of soman and atropine, HI-6 (125 mg/kg) caused significant reactivation of soman-inhibited acetylcholinesterase in the pons-medulla and in the striatum but produced no significant reactivation in the cortex.

It has been hypothesized that the main reason for the failure of various oximes to provide a beneficial effect against soman poisoning was the rapid aging of the inhibitor–enzyme complex [10, 11]. The rate of aging of soman-inhibited acetylcholinesterase was examined *in vivo* in rats by assessing the degree of reactivation of acetylcholinesterase in the diaphragm and intercostal muscles. A dose of soman (110 μg/kg) equivalent to a 24-hr LD_{50} was used to poison rats which had been pretreated with atropine (17.4 mg/kg). This dose of soman allowed some animals to survive for the required length of time before they received the HI-6. All animals were killed 3 hr after receiving soman. The results in Table 5 demonstrate that, 3 hr after receiving one LD_{50} of soman, the intercostal muscle acetylcholinesterase activity was not inhibited significantly, whereas the diaphragm acetylcholinesterase was inhibited by 49%. HI-6 was capable of reactivating the soman-inhibited acetylcholinesterase when applied up to 30 min after soman. These results demonstrate that the rate of aging of soman-inhibited acetylcholinesterase was not as fast as that previously found *in vitro* or *in vivo*. It was also found that the soman IC_{50} for acetylcholinesterase in the diaphragm and intercostal muscle was 0.66 ± 0.13 nM ($\bar{x}$ ± S.D.; N = 4) and 0.64 ± 0.32 nM ($\bar{x}$ ± S.D.; N = 4) respectively. These results were not significantly different.

DISCUSSION

Previous work from this laboratory on the mechanism of the therapeutic action of HI-6 versus soman poisoning revealed that HI-6 would completely protect mice; however, there was no evidence of reactivation of soman-inhibited acetylcholinesterase in the CNS [12]. It is possible that, if a small amount of reactivation were occurring in a very specific area of the brain, this would not be detectable in a whole brain homogenate. Therefore, further studies were carried out, using a larger rodent species, where we could examine discrete areas in the CNS and the periphery. Also, a more sensitive acetylcholinesterase assay was employed using [^{14}C]acetylcholine as the substrate.

The results of this study demonstrate that HI-6 (125 mg/kg) produced significant reactivation *in vivo* of soman-inhibited acetylcholinesterase in the respiratory musculature (diaphragm and intercostal muscles) but not in the CNS following soman (287 μg/kg) poisoning. Rats that survived soman

Table 5. Rate of aging of soman *in vivo* in the rat

Group	Intercostals	% Control	Diaphragm	% Control
Control	1.05 ± 0.06*	100	0.82 ± 0.12	100
Soman + atropine†	0.98 ± 0.15	93	0.42 ± 0.12	51
HI-6 at:‡				
0 min	1.09 ± 0.14	103	0.81 ± 0.14	99
5 min	1.01 ± 0.16	96	0.84 ± 0.18	102
10 min	1.04 ± 0.13	99	0.69 ± 0.09	84
15 min	0.89 ± 0.28	85	0.63 ± 0.27	76
30 min	1.14 ± 0.15	109	0.73 ± 0.21	89

* Values are nmoles acetylcholine hydrolysed · (mg tissue)$^{-1}$ · min^{-1} (mean ± S.D., N = 4).

† Rats were injected with soman (110 μg/kg, s.c.) 30 min after receiving atropine (17.4 mg/kg, i.p.). Rats were killed 3 hr after soman administration.

‡ Rats were treated as in † except that they received HI-6 (125 mg/kg, i.p.) either immediately after soman ("0" time) or at various times (5, 10, 15 or 30 min) after soman. Rats were killed 3 hr after soman injection.

poisoning (287 μg/kg) following HI-6 therapy had brain acetylcholinesterase levels which were not markedly different from those that had died following administration of HI-6. Since HI-6 is a bisquaternary ammonium compound, it was possible that the failure to produce reactivation of soman-inhibited acetylcholinesterase in the CNS had been due to its inability to cross the blood–brain barrier. However, it was found that *in vivo* HI-6 produced significant reactivation of sarin-inhibited brain acetylcholinesterase indicating, by indirect means, that HI-6 gained access to the CNS across the blood–brain barrier. Perhaps in the case of soman (287 μg/kg) poisoning not enough HI-6 entered the CNS to cause reactivation. In additional experiments addressing this question, the dose of soman was reduced to 110 μg/kg. When HI-6 (125 mg/kg) was administered therapeutically following soman (110 μg/kg), reactivation of soman-inhibited acetylcholinesterase was found in the striatum and pons-medulla. The above results suggest that at higher doses of soman (287 μg/kg) HI-6 provides its protective action by reactivation of soman-inhibited acetylcholinesterase in the respiratory musculature suggesting that the primary lethal lesion in soman (287 μg/kg) poisoning is peripheral. HI-6 did not produce significant reactivation of tabun-inhibited acetylcholinesterase.

In previous studies, it was reported that soman-inhibited acetylcholinesterase aged very rapidly *in vitro* (<2 min [10, 13]; 5.33 min [10]; 2.2 min [11]; <2 min [14]) and *in vivo* (16 min [15]; 5.55 min [10]; 1.5 to 5 min [14]). However, in this study soman-inhibited rat diaphragm acetylcholinesterase was still susceptible to reactivation by HI-6 *in vivo* for up to 30 min after soman administration. This result might account for the return of tetanic tension found by Smith and Muir [16] in guinea pigs poisoned by soman and given HS-6 and suggests that *in vivo* the rate of aging of soman-inhibited acetylcholinesterase is much slower than that found under *in vivo* conditions using conventional oximes. The rate of aging studies suggested that there was a difference in the sensitivity of intercostal and diaphragm muscle acetylcholinesterase to inhibition by soman. The intercostal muscle acetylcholinesterase was more resistant to inhibition by sarin, tabun and soman. However, *in vitro* studies showed that there was not a significant difference in the IC_{50} of acetylcholinesterase in the diaphragm or intercostal muscle produced by soman. Alternatively, a difference in distribution of soman to the diaphragm and intercostal muscles could account for the difference. This differential inhibition of diaphragm and intercostal muscle acetylcholinesterase *in vivo* may account for the observations by De Candole *et al.* [17] that "the chest muscles are less affected, so that chest movements of some magnitude may appear before and until the central inhibition is complete". In the unanesthetized rat, the diaphragm is the primary muscle in respiration. I found that severing both phrenic nerves in the neck region resulted in the death of all rats (J. G. Clement, unpublished observation) demonstrating that, if neuromuscular transmission in the diaphragm is imparied, death will ensue. Previous reports showed in anesthetized animals that neuromuscular transmission to the diaphragm of guinea pig [18] and rat [19] appeared to be much less affected when respiratory arrest occurred, suggesting a direct effect of soman in the central respiratory centre. The previous studies [18, 19] employed models assessing the functionality of the neuromuscular junction. I suggest that perhaps it is too rigorous a test, in that it appears to show function when in fact in the unanesthetized poisoned animal the neuromuscular junction could be blocked.

I propose that in the rat (and probably mouse) the mechanism of the therapeutic affect of HI-6 on soman poisoning is due to its high reactivating potency versus soman-inhibited acetylcholinesterase [20] in the diaphragm, specifically, combined with its low toxicity. The lack of, or low, antidotal actions of the other oximes is due to their relatively low reactivating potency combined with their relatively high toxicity. Therapeutic concentrations of these oximes to cause reactivation of soman-inhibited acetylcholinesterase cannot be obtained *in vivo* without reaching a toxic level first. Also, it is not unreasonable to assume that the mechanism of the lethality produced by soman is different depending upon the degree of poisoning and that possibly the mechanism of the therapeutic action of HI-6 may differ as well.

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