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REACTIVATION OF NERVE AGENT INHIBITED HUMAN ACETYLCHOLINESTERASES BY HI-6 AND OBIDOXIME

GERTRUD PUU, ELISABET ARTURSSON and GÖRAN BUCHT
Division of Experimental Medicine, National Defence Research Institute, Department 4, S-901 82 Umeå, Sweden

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Abstract—Acetylcholinesterase was purified from human caudate nucleus and skeletal muscle. The enzyme preparations were used to study aging and reactivation by HI-6 and obidoxime after inhibition by soman and its isomers. HI-6 was found to be the most potent reactivator. For both enzyme preparations a higher reactivatability and a higher rate of aging were observed after inhibition by C_+-soman than after inhibition by C_--soman. Aging was retarded by propidium diiodide. Reactivation by the two oximes was also studied after inhibition by tabun, sarin and VX. Tissue homogenates were used for this part of the work. Our conclusion is that HI-6 is superior to obidoxime for human acetylcholinesterases inhibited by soman and sarin, while obidoxime is better towards tabun-inhibited enzyme.

The current therapy for intoxications caused by acetylcholinesterase (EC 3.1.1.7) inhibitors of the organophosphate type consists of atropine and an oxime, such as pralidoxime ((2-hydroxyiminomethyl)-1-methyl pyridinium chloride) or obidoxime (1,1′-(oxybis(methylene))bis-(4-hydroxyiminomethyl-pyridinium dichloride). Atropine acts as a blocker at muscarinic acetylcholine receptors and thus protects against the excess of acetylcholine formed due to inhibiting the acetylcholine hydrolyzing enzyme. The oxime acts as a reactivator, restoring enzyme activity by splitting the bound organophosphorus residue from the enzyme.

Such a treatment is beneficial against poisoning by several organophosphorus insecticides [1–3] and also, as judged from animal experiments, by some of the highly toxic substances known as nerve agents. The efficacy is, however, very varying. A combination of atropine and any of the two oximes mentioned gives a good protection against sarin (isopropyl methylphosphonofluoridate) and VX (ethyl S-2-diisopropylaminoethylphosphonothiolate), while only the combination with obidoxime is effective against tabun (ethyl *N*,*N*-dimethylphosphoramidocyanidate). For intoxications caused by the fourth of the classical nerve agents, soman (1,2,2-trimethylpropyl methylphosphonofluoridate) none of the oximes in current use is of any therapeutic value. This lack of effect has been explained mainly by the rapid "aging" of soman-inhibited acetylcholinesterase, i.e. the process in which the bound inhibitor is dealkylated, resulting in an inhibitor–enzyme complex unsusceptible to oxime attack [4, 5]. There are, however, also opinions claiming that not only aging but also steric factors are of importance for the inability of conventional oximes to regenerate free enzyme after soman inhibition [6–8].

Such a view is supported by recent works showing that some bispyridinium mono-oximes, synthesized by Hagedorn and her co-workers, do give protection against soman, in several animal species ([9–14], for recent review see [15]). Some of these oximes have receptor blocking properties [16–18]. However, both *in vivo* [16, 19] and *in vitro* [20–22] studies indicate that reactivation is the main mode of action, especially for the most promising of these oximes, HI-6 (1-(2-hydroxyiminomethylpyridinium) - 1 - (4 - carboxyamido - pyridinium)dimethylether dichloride).

The efficacy of reactivation is not only determined by the structures of the inhibiting organophosphorus compound and the oxime but also on enzyme species [8, 22]. It is thus important to perform reactivation experiments with acetylcholinesterase of human origin. De Jong and Wolring have used human erythrocyte enzyme and studied reactivation after soman inhibition by a great number of oximes, with encouraging results with HI-6 [22]. We have now studied reactivation of acetylcholinesterase from tissues of importance for the toxic action of the nerve agents, i.e. from skeletal muscle and from brain (caudate nucleus). We have compared the effects of HI-6 and obidoxime after inhibition by sarin, tabun, VX and soman. The interactions between a purified enzyme from caudate nucleus, HI-6 and soman, including its stereoisomers, were investigated in greater detail.

MATERIALS AND METHODS

Tabun, sarin, VX, C_+, C_- and racemic soman, all of high purity as determined by ^{1}H- and ^{31}P-NMR-analysis were synthesized at the Chemistry Department of this institute. Obidoxime and pralidoxime chloride were from commercial sources, while HI-6 was a gift from Professor I. Hagedorn, University of Freiburg, F.R.G. Propidium diiodide was from Sigma Chem Co; edrophonium bromide from Roche Products. Human tissues were obtained from the University Hospital, Umeå. Corpses were transported to a cold-room (+4°) not later than 4 hr after death. Tissues were removed at autopsy and immediately frozen at −70° and kept at that temperature until use.

Preparation of homogenates of caudate nucleus. A

1.2 g tissue sample was homogenized in 10.8 ml sodium phosphate buffer, pH 7.4, containing 0.1% Triton X-100. The suspension was then stirred, and 1 g of dry Sephadex G-25 added to concentrate the solution and to remove some low molecular weight compounds. Stirring was continued for 2 hr. The suspension was centrifuged for 20 min at 20,000 *g*, and the supernatant was diluted with buffer four times. All steps were performed at 2–4°.

Preparation of homogenates of skeletal muscle (m. vastus lateralis). A 20.4 g tissue sample was cut into smaller pieces and then homogenized in a Waring blender with 100 ml 10 mM Tris–HCl, 10 mM $MgCl_2$, 1 M NaCl and 1% Triton X-100, pH 7.4, followed by homogenization with a Potter–Elvehjem homogenizer. The suspension was diluted to 200 ml, 10 g dry Sephadex G-25 was added and the preparation was stirred for 20 hr. After centrifugation at 20,000 *g* for 20 min the supernatant was poured through cheesecloth and centrifuged again. The whole procedure was done in a cold-room.

Reactivation in homogenates. Acetylcholinesterase in homogenates of each tissue was inhibited, to 80–95%, with tabun, sarin and VX, respectively, for 30 min at pH 7.4, 22°. The concentrations of nerve agent were: tabun 1×10^{-7} M (muscle) and 2×10^{-7} M (caudate); sarin 5×10^{-8} M (both tissues); VX 1×10^{-8} M (muscle) and 5×10^{-9} M (caudate). To start reactivation an equal volume of obidoxime- or HI-6-containing 0.1 M sodium phosphate buffer, pH 7.4, was added. Final concentration of oxime was 0.1 mM. Reactivation proceeded for 4 hr at room temperature. Percentage reactivation was calculated as suggested by Keijer *et al.* [23].

In some experiments acetylcholine iodide (0.5–50 mM) was included in the reactivation incubation medium.

Preparation and regeneration of affinity gel. Sepharose 4 B was chosen as solid support, to which a long spacer and the ligand 1-(*N*,*N*,*N*,-trimethylammonium)-6-hexylamine bromide were coupled, as described by Hopff *et al.* [24].

The affinity gel was regenerated after each run by washing on a glass filter with 10 mM decamethonium bromide, followed by 1 M sodium chloride and finally 0.5 g of the ligand dissolved in 100 ml water. The gel was then equilibrated with the buffer used for enzyme purification.

Partial purification of acetylcholinesterase from caudate nucleus. A 7 g tissue sample was disintegrated in a glass homogenizer with a Teflon pestle in 40 ml ice-cold 10 mM Tris–HCl, pH 7.4, 144 mM NaCl, 1% Triton X-100 [25]. The suspension was stirred for 3 hr in a cold-room and centrifuged for 1 hr at 100,000 *g*. The supernatant was added to the affinity gel. This mixture was gently stirred overnight in a cold-room, sucked off and washed with the high ionic strength buffer on a glass filter. The gel was packed in a small column (0.9 × 12 cm) and acetylcholinesterase was eluted with 10 mM decamethonium bromide. Enzyme-containing fractions were pooled and dialysed against 0.1 M sodium phosphate buffer, pH 7.4, 0.05% Triton X-100.

Partial purification of acetylcholinesterase from skeletal muscle. The tissue was treated as described for preparation of homogenate but 5 mM sodium phosphate buffer, pH 6.9, containing 1% Triton X-100 was used as homogenization medium. The mechanical disintegration was followed by sonication (Branson sonifier) for 2 min. The procedure then followed the one described for caudate enzyme, with the exception that the low ionic strength buffer used for homogenization was used throughout. The final dialysis was, however, against 0.1 M sodium phosphate buffer, pH 7.4, 0.05% Triton X-100.

Acetylcholinesterase assay. Ellman's procedure was used in all experiments [26], with 1 mM acetylthiocholine iodide as substrate and in the presence of 0.1% Triton X-100. The measurements were done on an automatic enzyme analyzer (Clinicon Corona, Bromma, Sweden) in duplicates.

Reactivatability and aging of soman-inhibited enzymes. The highly purified enzyme preparations, with addition of 0.5% bovine serum albumin, were used to determine rate constants of aging and maximal reactivation of soman-inhibited enzymes. The experiments were run as described recently for plaice cholinesterase [8]. However, because of the more rapid aging of the human enzymes, samples were withdrawn from the incubation mixture more frequently, usually once a minute.

RESULTS

Acetylcholinesterase in tissue homogenates: inhibition and reactivation

The nerve agents soman, sarin, tabun and VX were all found to be potent inhibitors of acetylcholinesterase in tissue homogenates. The relative order of potency was, for both tissues, soman > VX > sarin > tabun. No bimolecular reaction constants could be obtained, as the inhibition did not follow pseudo-first-order kinetics, probably due to binding and/or degradation of nerve agents by proteins or other material present in the homogenates.

For reactivation of enzyme in homogenate, inhibited to 80–98% by sarin, tabun and VX, respectively, 0.1 mM oxime was used. Both HI-6 and obidoxime were tested as reactivators. As seen in Table 1, these two oximes were good reactivators of VX-inhibited enzyme in muscle as well as in caudate homogenates. Sarin-inhibited enzyme in both tissues was easily reactivated by HI-6. Obidoxime was also found to have a good reactivating effect in the caudate homogenate but was inferior to HI-6 in the muscle preparation. Obidoxime was, on the other hand, much better than HI-6 as reactivator towards tabun-inhibited acetylcholinesterase. Tabun-inhibited enzyme in the muscle preparation seemed to be somewhat susceptible to reactivation by HI-6.

We also tested whether the presence of acetylcholine had any influence on the reactivation after inhibition by sarin or VX. Acetylcholine (0.5, 1, 2 or 50 mM) was added simultaneously with oxime (0.1 mM obidoxime or HI-6). We found no significant effect on reactivation degree.

Purification of enzymes

As the cholinesteratic activity in the tissue homogenates was too low to permit studies on aging and reactivatability of soman-inhibited enzyme, an enrichment procedure was necessary. We used affin-

Table 1. Percentage reactivation by HI-6 and obidoxime of nerve agent inhibited acetylcholinesterase in tissue homogenates

Nerve agent	Caudate		Muscle	
	Obidoxime	HI-6	Obidoxime	HI-6
Tabun	76 (±9)	4 (±1)	73 (±20)	12 (±1)
Sarin	84 (±13)	100 (±1)	44 (±9)	102 (±9)
VX	82 (±3)	86 (±5)	80 (±6)	80 (±5)

Acetylcholinesterase in tissue homogenates was inhibited to 80–98% by nerve agent. Oxime was added, final concentration 0.1 mM, and reactivation proceeded at room temperature (22°) for 4 hr, pH 7.4. Standard deviations are shown in parentheses.

ity chromatography with 1-(*N*,*N*,*N*-trimethylammonium)-6-hexylamine as ligand and decamethonium as eluant. This procedure worked very well with the enzyme from caudate nucleus. The recovery was 65% and the purification factor was found to be about 6000.

This simple and rapid enrichment procedure, developed for the brain enzyme, could not be used directly for the muscle enzyme. Acetylcholinesterase from this tissue did not bind to the affinity gel at high ionic strength. By performing all steps at low ionic strength we could also get an enrichment of acetylcholinesterase from this tissue, but as the material obtained was both qualitatively and quantitatively inferior to the brain enzyme, we preferred to work mainly with the latter preparation.

Reactivatability of soman-inhibited acetylcholinesterase by various oximes

The reactivatability of unaged soman-inhibited enzyme was determined from aging curves by extrapolating to time zero. The procedure is illustrated in Fig. 1, showing the time-dependent loss of reactivatability of soman-inhibited caudate acetylcholinesterase with 0.1 mM HI-6 (upper curve) and obidoxime (lower curve) as reactivators, and in Fig. 2. We found that HI-6 is a much better reactivator of soman-inhibited acetylcholinesterase of human origin than the conventional oximes obidoxime and PAMCl (Table 2).

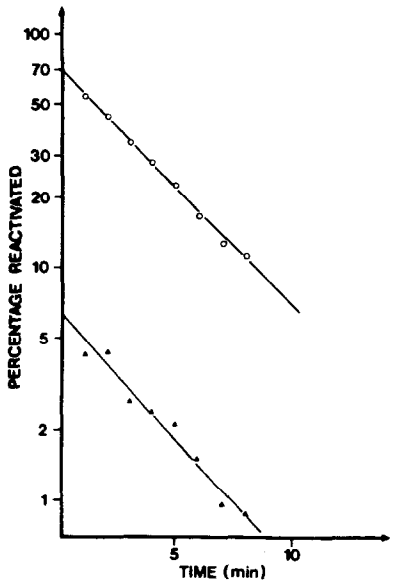

Fig. 1. Aging curve for soman-inhibited acetylcholinesterase from human caudate nucleus. Inhibition and aging in 0.1 M sodium phosphate buffer, pH 7.4, at 22°. Reactivation with 0.1 mM obidoxime (lower curve) and 0.1 mM HI-6 (upper curve) for 4 hr at pH 7.4. Initial (maximal) reactivatable enzyme is defined as the intercept on the ordinate.

The maximal reactivation obtained by an oxime is of course highly dependent on concentration. In our standard conditions we used 0.1 mM HI-6. A tenfold lower concentration also gave substantial reactivation (28%) while 1 μM HI-6 resulted in a reactivation degree comparable to the one obtained by 0.1 mM obidoxime (i.e. 6%).

Table 2. Percentage maximal reactivation of soman-inhibited acetylcholinesterases by 0.1 mM HI-6, obidoxime and PAMCl

Oxime	Muscle AChE	Caudate AChE
HI-6	74.9	70.9
Obidoxime	Not detectable	6.4
PAM	Not determined	5.6

Reactivation at 25° for 4 hr at pH 7.4.

Reactivatability and aging of enzymes inhibited by racemic, C_+- and C_--soman

Racemic soman contains four stereoisomers, as the molecule has two chiral centres, the phosphorus atom and the α-carbon atom in the pinacolyl group. We performed experiments on soman, resolved at the α-carbon. Thus the preparation of C_+-soman and C_--soman contains C_+P_+ plus C_+P_- and C_-P_+ plus C_-P_-, respectively, but only the P_--forms are of importance for inhibition [27, 28]. We found, for enzyme from both tissues, that C_+-soman inhibited acetylcholinesterase ages more rapidly and is more reactivatable than C_--soman inhibited enzyme (Figs 3, 4, Table 3). We also noted that the enzyme preparation from skeletal muscle, inhibited by any of the two isomers, was more susceptible to oxime attack than correspondingly inhibited enzyme from caudate nucleus (Table 3). Despite this higher degree of reactivation after inhibition by the isolated isomers, the muscle and brain enzyme had the same reactivatability after inhibition by the racemate. This seemingly contradictory result might have its explanation—and some support for this explanation is given by the aging profiles for the different enzyme–inhibitor complexes—in an unequal contribution of C_+- and C_--soman in the racemate in inhibiting

Table 3. Aging rate constants and percentage reactivatable enzyme by 0.1 mM HI-6 after inhibition of acetylcholinesterase from skeletal muscle and caudate by racemic, C_+- and C_--soman

	Muscle AChE		Caudate AChE	
	k_{obs} (min^{-1})	Reactivation (%)	k_{obs} (min^{-1})	Reactivation (%)
Racemic soman	0.262 (0.040)*	74.9 (2.3) (3)†	0.275 (0.066)	70.9 (5.2) (3)
C_+-soman	0.343 (0.042)	90.6 (3.4) (2)	0.309 (0.051)	70.8 (7.9) (3)
C_--soman	0.214 (0.040)	58.4 (2.6) (3)	0.180 (0.049)	31.1 (3.6) (3)

* Values given as means, standard deviations in parentheses.
† Number of experiments.

the two enzymes. While C_+-soman in the racemate seems to dominate inhibition of the caudate enzyme, the two isomers seem to be of equal importance for inhibition of the muscle enzyme.

Influence of quaternary nitrogen compounds on aging rate

Some quaternary compounds, e.g. SAD-128 [29], (2-(4-pyridyl)ethyl)diethylmethylammonium iodide [30] and (+)tubocurarine [8], at high concentrations, have been shown to slow down the rate of aging. Although not directly shown, it is suggested that retardation is caused by an allosteric modulation of the enzyme–inhibitor complex.

We tested the effect of 1 μM edrophonium, specific for the active site anionic binding site and 10 μM propidium (3,8-diamino-5,3′-diethylamino-*n*-propyl-6-phenylphenanthridium), which binds to the peripheric anionic site of acetylcholinesterase [31]. The substance under study was added to the enzyme solution before soman and was thus present during both inhibition and aging. The concentration was reduced by a factor of 40 during reactivation.

We found that while edrophonium ions had no effect on aging rate or reactivatability, the presence of propidium ions did influence the rate of aging but not the degree of reactivation of soman-inhibited caudate acetylcholinesterase (Fig. 5). We also found that propidium ions had the most pronounced retarding effect on C_+-soman inhibited enzyme (Table 4).

DISCUSSION

Acetylcholinesterases from various species generally behave very similarly towards substrates and inhibitors, suggesting a great homology in the active site of the enzymes. However, other studies, such as immunological crossreactivity with use of monoclonal antibodies [32, 33], indicate differences in amino acid sequences and/or three-dimensional structure. Soman inhibition and reactivation studies by de Jong and Wolring [22] also suggest that the active sites are heterogeneous.

How similar are acetylcholinesterases from different tissues within a species? The monoclonal antibodies raised by Fambrough *et al.* [32] against human erythrocyte enzyme crossreacted with acetylcholinesterase in human neuromuscular junction but only to a limited extent with caudate nucleus enzyme. Brimijoin *et al.*, on the other hand, raised monoclonal antibodies against the same enzyme which recognized the caudate nucleus acetylcholinesterase very well [33]. Recently, de Jong and Wolring have reported that three enzymes from mice have, after soman inhibition, very similar reactivation and aging properties [34].

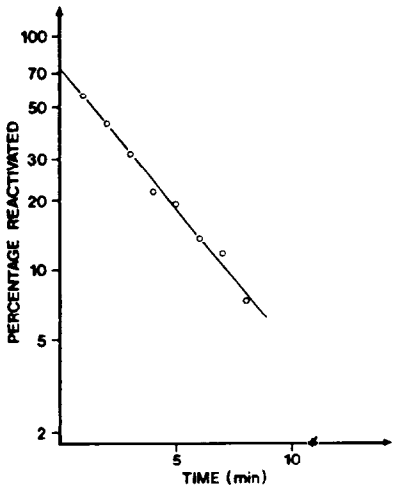

Fig. 2. Aging curve for soman-inhibited acetylcholinesterase from human skeletal muscle. Reactivation with 0.1 mM HI-6.

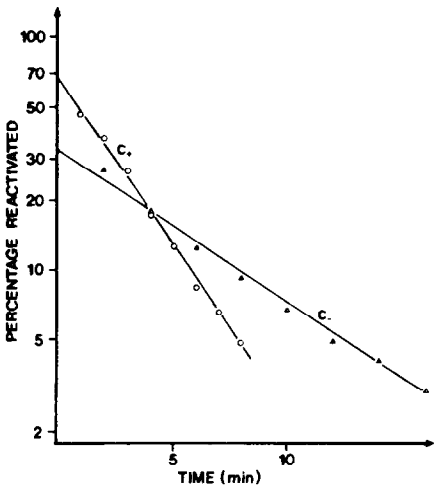

Fig. 3. Aging curves for acetylcholinesterase from caudate nucleus, inhibited by C_+- and C_--soman, respectively, and reactivated by 0.1 mM HI-6.

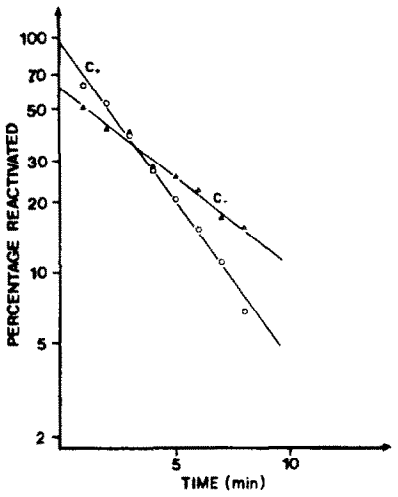

Fig. 4. Aging curves for acetylcholinesterase from skeletal muscle, inhibited by C_+- and C_--soman, respectively, and reactivated by 0.1 mM HI-6.

We also found when comparing human muscle and caudate nucleus acetylcholinesterases that the two enzymes have in most respects the same properties. The sensitivity towards the four nerve agents studied was found to be the same for the two enzymes. Reactivation degrees after inhibition, with use of different oximes, were in most cases equivalent, and, furthermore, were in agreement with results reported for human erythrocyte enzyme [13, 22]. A comparison between the two enzymes of the aging rates after inhibition by soman or its stereoisomers also suggest that the active sites are very much the same.

Some observations point towards dissimilarities. We found that the muscle enzyme could not bind to the affinity gel at high ionic strength, in contrast to the brain enzyme. We have had the same problem

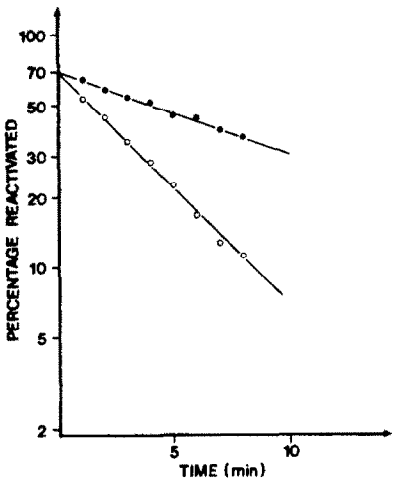

Fig. 5. Aging curves for soman-inhibited acetylcholinesterase from caudate nucleus in the presence (upper curve) and absence (lower curve) of 10 μM propidium diiodide during inhibition and aging.

Table 4. Aging rate constants of caudate acetylcholinesterase inhibited by racemic, C_+- and C_--soman in the presence of 10 μM propidium diiodide

	k_{obs} (min^{-1})
Racemic soman	0.101 (0.027) (3)
C_+-soman	0.107 (0.032) (2)
C_--soman	0.116 (0.028) (3)

when purifying acetylcholinesterase from human erythrocytes. We were also surprised to find a rather low (44%) degree of reactivation by HI-6 of sarin-inhibited muscle enzyme and for the same enzyme as unexpected high (12%) degree of reactivation after tabun inhibition. The experiments on soman racemate and isomers also indicate some differences in active site properties of the two enzymes. Firstly, the brain enzyme incubated with racemic soman seemed to be almost exclusively inhibited by the C_+P_- form, while the two isomers seemed to be of equal importance for inhibition of the muscle enzyme. Secondly, we found that the muscle enzyme, inhibited by the isolated isomers, had higher reactivatabilities than the caudate enzyme counterparts.

We found that C_+-soman inhibited enzyme, from both tissues, was more susceptible to oxime attack than C_--soman inhibited enzyme. That the configuration around the α-carbon in the pinacolyl moiety of the enzyme-bound soman is important for reactivation has been shown for several enzyme species, including plaice [6], electric eel [22] and several mammalian acetylcholinesterases [22, 23]. C_+-soman has in all cases, and regardless of oxime used for reactivation, been shown to give the most readily reactivated enzyme–inhibitor complex. While de Jong and Wolring reported [22] that the rate of aging, i.e. the process in which the pinacolyl moiety is split off from the bound soman molecule, is independent of the configuration around the α-carbon, we have come to the opposite result, both for plaice [8] and human enzymes. We have also found that it is possible, by addition of suitable substances, to modulate the enzyme–inhibitor complexes in such a way that the aging rates become independent of soman configuration. One example is given in the present work. A low concentration of propidium, which binds to the peripheral anionic site of acetylcholinesterase, retards the aging and results in the same aging rate constant for C_+-, C_-- and racemic soman.

In conclusion, we have found that HI-6 is a good reactivator of sarin-, VX- and soman-inhibited acetylcholinesterase from human caudate nucleus and skeletal muscle. The high rate of aging, at least *in vitro*, of soman-inhibited human acetylcholinesterase might, however, limit the therapeutic value of HI-6 against soman intoxications. Whether the soman–enzyme complex ages rapidly also in more *in vivo*-like conditions, as well as the relative contribution of the different stereoisomers for inhibition, will be further studied.

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