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Two possible orientations of the HI-6 molecule in the reactivation of organophosphate-inhibited acetylcholinesterase

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Abstract

The inhibition of acetylcholinesterase (AChE) by organophosphorus compounds (OPs) causes acute toxicity or death of the intoxicated individual. One group of these compounds, the OP nerve agents, pose an increasing threat in the world due to their possible use in the battlefield or terrorist acts. Antidotes containing oxime compounds to reactivate the inhibited enzyme are highly valued for treatment against OP poisoning. One of these reactivators, HI-6, was shown to be significantly more effective in treating soman toxicity than other oximes, such as 2-PAM, TMB4, and obidoxime. However, HI-6 was less effective in reactivating AChE inhibited by the OP pesticide, paraoxon. In this study, the mechanism for HI-6-induced reactivation of OP–AChE conjugates was investigated using mouse mutant AChEs inhibited with different OPs including organophosphate paraoxon, and several methylphosphonates. Results indicate that the HI-6 molecule may assume two different orientations in the reactivation of AChE inhibited by organophosphate and Sp methylphosphonates. These conclusions were further corroborated by reactivation studies using an analog of HI-6 in which the bispyridinium moieties are linked by a methylene bridge rather than an ether oxygen.

Keywords: Acetylcholinesterase; Reactivation; Organophosphates; HI-6; Oxime

The inhibition of acetylcholinesterase (AChE; E 3.1.1.7) by organophosphorus compounds (OPs) causes acute intoxication. Chemical nerve agents pose an increasing threat in the world due to their possible use in battlefield or terrorist acts. At the same time, OP pesticides, such as paraoxon and its analogs, are being used throughout the world in agriculture causing hundreds of thousands of intoxication cases each year. At the present time, available antidotes against OP poisoning use oximes, such as 2-PAM, obidoxime, TMB4, and HI-6, to reactivate the inhibited enzyme. Of the nerve agents, soman is the greatest challenge since both the rapid aging of the soman-inhibited enzyme (de-alkylation) and the bulkiness of the pinacolyl residue prevent efficient reactivation of the inhibited enzyme by most oximes. HI-6, an oxime first synthesized in the early 1970s, was shown to be a very effective antidote in reactivating soman-inhibited AChE. But compared with other oximes, HI-6 was much less effective in reactivating paraoxon- or methylparaoxon-inhibited AChE, making it unsuitable for the treatment of OP pesticide poisoning [1,2]. The mechanism for the selectivity of HI-6 in reactivating soman-inhibited enzyme over paraoxon-inhibited enzyme is not yet known.

Several recent studies have demonstrated the successful use of site-directed mutagenesis to explore the interactions between OP-conjugated AChE and oxime reactivators

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Abbreviations: AChE, acetylcholinesterase; MoAChE, recombinant mouse wild-type acetylcholinesterase; OPs, organophosphorus compounds; POX, phosphonyl or phosphoryl oximes; DEPQ, 7-(*O*,*O*-diethylphosphinyloxy)-1-methylquinolinium methyl sulfate; MEPQ, 7-(*O*-ethyl methylphosphinyloxy)-1-methylquinolinium iodide; iPrMP, isopropyl methyl phosphonothiochline; 2-PAM, 2-[hydroxyimino methyl]-1-methylpyridinium chloride; TMB4, 1,1′-trimethylene bis(4-hydroxyimino methyl) pyridinium dichloride; obidoxime (toxogonin), 1,1′-(oxybismethylene)bis[4-(hydroxyimino)methyl] pyridinium dichloride; HI-6, 1-(2-hydroxyiminomethyl-1-pyridinium)-1-(4-carboxy-aminopyridinium)-dimethyl ether hydrochloride.

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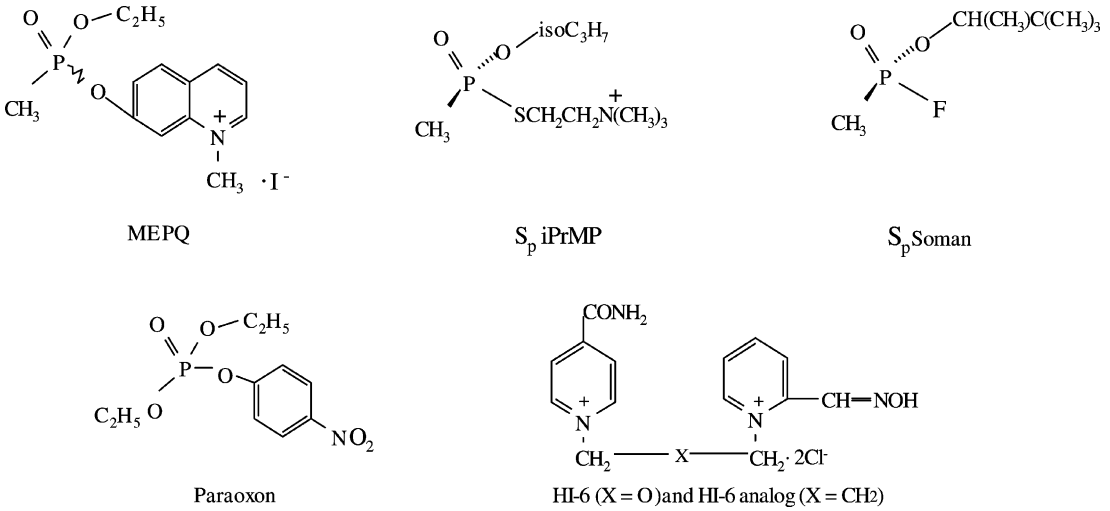

Fig. 1. Structures of organophosphate, organophosphonates, and oximes used in the study.

[3–6]. However, an important issue in the study of reactivation kinetics is the possibility of phosphoryl oxime (POX) inhibition of the reactivated enzyme during the reactivation process that may prevent the accurate determination of reactivation rate constants [6–8]. In our previous studies, we demonstrated that of the four oximes, 2-PAM, TMB4, obidoxime, and HI-6, HI-6 was the only one that did not show any complications due to POX inhibition during the reactivation of AChE inhibited by either organophosphonate (MEPQ) or organophosphate (DEPQ). Therefore, we conducted reactivation kinetic studies with HI-6 using mouse wild-type and mutant AChEs inhibited by different OP compounds, including organophosphate paraoxon, racemic methylphosphonate MEPQ, Sp enantiomeric iPrMP, and SpSc soman (Fig. 1). The results of our study highlight some significant differences between the interactions of HI-6 with organophosphate- and methylphosphonate-inhibited AChEs during reactivation, which are also supported by reactivation studies using an analog of HI-6 that is devoid of the ether chain oxygen.

1. Materials and methods

1.1. Materials

MEPQ and Sp iPrMP were prepared by the method reported previously [9,10]. HI-6 was obtained from the Division of Experimental Therapeutics, Walter Reed Army Institute of Research. HI-6 analog was synthesized by method deployed in oxime synthesis and the structure confirmed by proton NMR. Recombinant mouse wild-type AChE (MoAChE) and mutant enzymes were prepared by the method previously reported [11,12]. Organophosphate paraoxon was purchased from Sigma Chemical Co. Bio-Spin 6 chromatography columns were purchased from Bio-Rad Laboratories. Other chemicals were from commercial resources.

1.2. AChE assay

AChE activity was determined spectrophotometrically by the method of Ellman *et al.* [13]. The assay mixture contained 1 mM acetylthiocholine (ATC; or 30 mM ATC in the case of W86A mutant AChE) as the substrate and 0.5 mM 5,5-dithiobis-(2-nitrobenzoic acid) (DTNB) in 50 mM sodium phosphate buffer, pH 8.0. All measurements were performed at 25°.

1.3. Determination of the reactivation rate constants of OP-inhibited enzyme by oximes

AChE–OP complexes were prepared by adding greater than the stoichiometric amount of OP to wild-type or mutant MoAChEs in a total volume of 100 μL and incubating for 20 min at 25°. Excess OP was removed from the complex using a Bio-Spin column 6. The enzyme conjugate was then diluted 10-fold with 0.05% BSA in 50 mM phosphate buffer, pH 8.0, containing the oxime. The final oxime concentration in the reactivation mixture ranged from 50 μM to 3 mM. At specified time intervals, 5–10 μL of reactivation mixture was withdrawn and diluted into the assay mixture to monitor change in enzyme activity. The first-order reactivation rate constant, k_{obs}, was determined by fitting the experimental data to equation for one-phase exponential association:

$$\%\,(E_{react})_t = A(1 - e^{-k_{obs} \times t})$$

A plot of k_{obs} vs. [oxime] was used to obtain the second-order reactivation rate constant as described by Ashani *et al.* [3]. Due to the chirality of the phosphorus in MEPQ, the phosphonyl conjugate enzyme consists of two enantiomers that are reactivated at different rates. The reactivation rate constants of the two species were calculated using a two-phase exponential association equation, assuming an equal distribution of the two species under the condition that a stoichiometric amount of MEPQ was used for the inhibition of AChE.

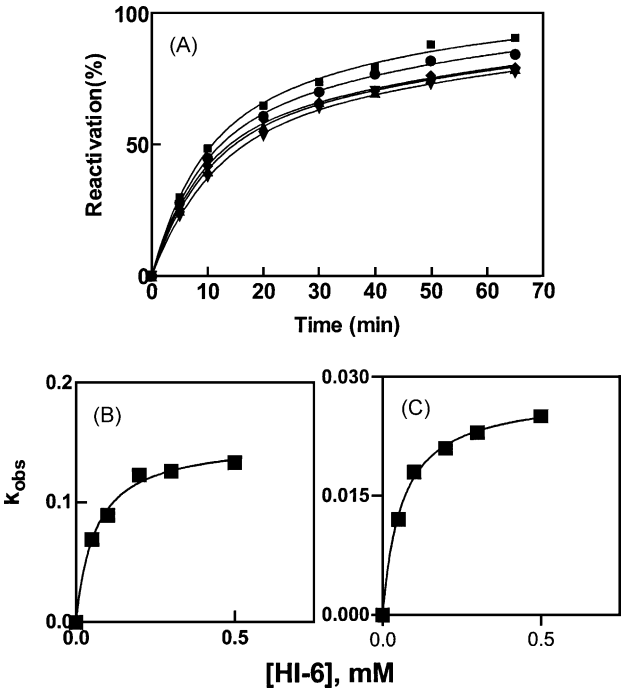

Fig. 2. Reactivation kinetics of MEPQ-inhibited wild-type MoAChE by HI-6. (A) Reactivation kinetics of one representative experimental data of MEPQ-inhibited wild-type MoAChE by HI-6 (0.05 to 0.5 mM); symbols represent the following concentrations of HI-6: (▼) 0.05 mM; (▲) 0.1 mM; (◆) 0.2 mM; (●) 0.3 mM; (■) 0.5 mM. (B) Secondary plot of the k_{obs} for the fast-reactivation species vs. [HI-6] for the second-order kinetic constants. (C) Secondary plot of the k_{obs} for the slow-reactivation species vs. [HI-6].

2. Results

2.1. Reactivation rate constants of OP-inhibited MoAChE by HI-6

As shown in Fig. 2, the reactivation data for MEPQ-inhibited wild-type MoAChE using five different HI-6 concentrations fit to the two-phase exponential association equation well. The secondary plot of k_{obs} vs. [HI-6] yields values of the second-order reactivation rate constants of 2700 and 462 M^{-1} min^{-1} for the fast- and slow-reactivation species, respectively. The second-order reactivation rate constants of wild-type MoAChE inhibited with other OPs determined using this method showed that these constants varied considerably depending on the structure of OP (Table 1). Sp iPrMP- and SpSc soman-inhibited AChEs, as well as the fast-reactivation species of MEPQ-inhibited AChE were reactivated several fold faster than paraoxon-inhibited AChE and the slow-reactivation species of MEPQ-inhibited AChE.

2.2. Effect of mutations on the reactivation of OP-inhibited MoAChE by HI-6

Mutations of F295 to Leu or F297 to Ile reduced the reactivation rate of the fast-reactivation species of MEPQ-inhibited enzyme about 2-fold, but the reactivation rates for the slow-reactivation species were reduced >300-fold for F297I AChE or to a negligible reactivation rate for F295L AChE. The reactivations of both the active anionic site mutant, W86A, and the peripheral anionic site mutant, W286A, were only reduced by a factor of 3–5 for the fast- and slow-reactivation species of MEPQ-inhibited enzyme. However, the mutation of another peripheral anionic site residue, Y124 to Gln, reduced the reactivation rate constant of the fast-reactivation species by 87-fold, and the slow-reactivation species by 24-fold. This mutation significantly reduced the HI-6-induced reactivation rate constants for both Sp iPrMP-inhibited enzyme (36-fold) and SpSc soman-inhibited enzyme (155-fold). Also, 1.4- to 3-fold increases in HI-6-induced reactivations were observed with Sp iPrMP and SpSc soman-inhibited F295L, F297I, and W86A with the exception of iPrMP-inhibited F297I, whose reactivation rate constant decreased slightly compared with wild-type enzyme.

The effects of these mutations on the reactivation rate constants of paraoxon-inhibited mouse AChEs were quite different from those inhibited by all the methylphosphonates studied. First, the reactivation rate of paraoxon-inhibited wild-type enzyme by HI-6 was 3- to 5-fold slower compared with Sp iPrMP- and Sp soman-inhibited AChEs or the fast-reactivation species of MEPQ-inhibited AChE, despite the fact that paraoxon-inhibited enzyme could be reactivated very easily by other bispyridinium oximes such as LüH6 [1,2]. Second, the reactivation rate constants of paraoxon-inhibited F295L and F297I AChEs displayed

Table 1
Second-order rate constants (k_r, M^{-1} min^{-1}) for the reactivation of OP-inhibited mouse wild-type and mutant AChEs by HI-6

Enzyme	MEPQ[a]	Sp iPrMP	SpSc soman[b]	Paraoxon
Wild-type	2700 (fast); 462 (slow)	2650	1751	590
F295L	1263 (fast); NA (slow)	3320[c]	5420	2.15
F297I	1325 (fast); 1.3 (slow)	2400[c]	3460	1.47
W86A[d]	947 (fast); 84 (slow)	3606	5245	22.6
W286A	673 (fast); 105 (slow)	913	732	151
Y124Q	31 (fast); 19 (slow)	74	11.3[e]	103

Data are mean values of two to four experiments with all reactivation experiments carried out at 25° in 50 mM PO_4 buffer, pH 8.0, except where indicated.

[a] The reactivation rate constants of MEPQ-inhibited enzymes were obtained by fitting the kinetic data to the two-component equation [3], and the maximal reactivation was more than 92% except with F295L, where only 55–62% reactivation was observed after 50 hr.

[b] Reactivation was carried out at 25° in 50 mM PO_4 buffer, pH 8.0, after inhibition in 50 mM Tris buffer, pH 9.5; maximal reactivation is from 86 to 95% except where indicated.

[c] Data cited from Wong *et al.* [5] where the reactivation rate constants were determined in 50 mM Tris–HCl buffer, pH 8.0.

[d] The activity assay for W86A was performed using 30 mM ATC instead of 1 mM.

[e] Data estimated from k_{obs} of a single concentration of HI-6 assuming that it is in the linear range of the saturation curve, maximal reactivation was 31%.

Table 2
Comparison of the second-order rate constants for the reactivation of OP-inhibited mouse wild-type AChE by HI-6 and its analog

OPs	k_r (M^{-1} min^{-1})	
	HI-6	HI-6 analog
MEPQ		
Fast-reactivation species	2700	544
Slow-reactivation species	462	291
Paraoxon	690	668

The second-order reactivation rate constants were determined as described in Table 1. Data were in k_r (M^{-1} min^{-1}).

drastic reductions (275- to 400-fold) compared with the wild-type enzyme, while this was not observed with most of the methylphosphonate-inhibited AChEs. Third, the reactivation rate constant of paraoxon-inhibited W86A AChE by HI-6 was 26-fold lower compared to wild-type AChE, a phenomenon not observed with any of the methylphosphonate–enzyme conjugates. Finally, the reactivation rate of paraoxon-inhibited Y124Q AChE by HI-6 showed only a 5-fold reduction instead of the 36- to 155-fold reductions observed with the Sp methylphosphonate–enzyme conjugates.

2.3. Comparison of the reactivation kinetics of MEPQ- and paraoxon-inhibited wild-type mouse AChEs by HI-6 and its analog

Reactivation studies with mutant enzymes showed that the HI-6-induced reactivation of AChEs inhibited by Sp methylphosphonate, but not by organophosphate paraoxon, displayed a significant dependence on the peripheral anionic site amino acid, Y124. Computer modeling by Ashani *et al.* [3] previously indicated that Y124 might interact with the ether oxygen through a hydrogen bond to facilitate the orientation of HI-6 during reactivation of MEPQ-inhibited AChE. Therefore, we conducted reactivation studies with an HI-6 analog to determine if the substitution of the ether oxygen by methylene group has any effect on the reactivation of different AChE–OP conjugates. Results in Table 2 show that absence of that oxygen significantly reduced the reactivation potency of the oxime for the fast-reactivation species of MEPQ-inhibited enzyme by 5-fold, but not the slow-reactivation species or paraoxon-inhibited enzyme.

3. Discussion

Studies on the reactivation of OP-inhibited AChE will not only enhance our understanding of the reactivation mechanism, but also help the design for better and more effective reactivators. Reactivation by oxime compounds is a process of neucleophillic reaction involving the deprotonized negative oxygen of the oxime group to attack the phosphorus atom of OP moiety in the active center of AChE, resulting in the departure of AChE from OP–enzyme conjugate (Fig. 3 depicted the binding relationship of MEPQ-conjugated AChE and HI-6 molecule). The search for better reactivator is important for improving the efficacy of antidotes against OP toxicity. HI-6, a bispyridinium oxime containing the structural features of 2-PAM, is a very powerful reactivator of nonaged soman-inhibited AChE [14]. Results of reactivation studies using AChE inhibited by MEPQ, a methylphosphonate,

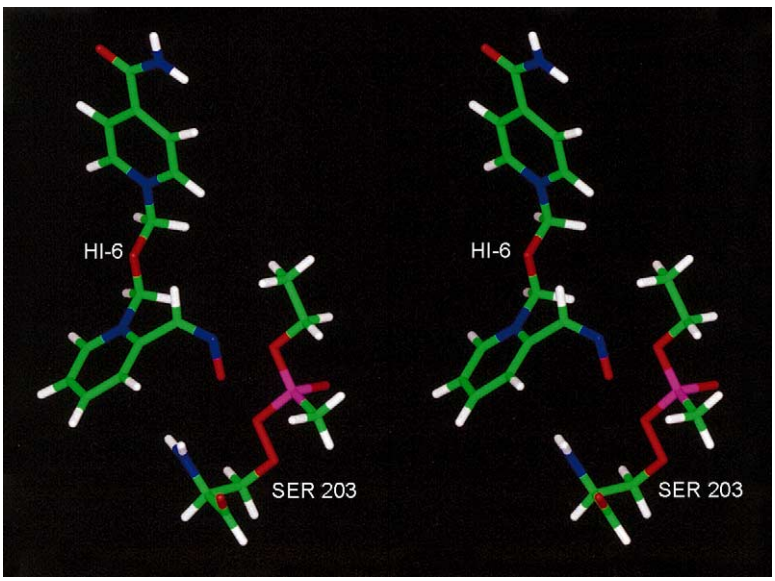

Fig. 3. Stereoview of relationship between MEPQ-inhibited AChE and oxime reactivator HI-6. The stereoview was made using Insight II (version 2000); the phosphorus atom of MEPQ is marked in pink, the methyl and ethoxyl groups are marked in green, and the oxygen of the oxime group of HI-6 is marked in red. The deprotonized negative oxygen of the oxime group approaches the phosphorus atom of Sp MEPQ residue in the active center of AChE to initiate neucleophilic attack, resulting in the departure of OP residue and generation of free enzyme.

suggested that the enhanced reactivation potency of HI-6 is due to the interaction of the second pyridinium ring with the peripheral anionic site of AChE which facilitates the orientation of HI-6 during reactivation [3]. However, other oximes containing the second pyridinium structure, such as obidoxime and TMB4, display very poor reactivation potencies for AChE inhibited by soman, also a methylphosphonate. Therefore, the mechanism for increased activity of HI-6 in reactivating soman-inhibited AChE is unclear.

In this study, we used three methylphosphonates with increasing size of the alkoxy groups, i.e. ethyl, isopropyl, and pinacolyl, and an organophosphate paraoxon, to form enzyme–OP conjugates for reactivation by HI-6. Two of these three methylphophonates (Sp iPrMP and SpSc soman) were pure Sp enantiomers and expected to yield a single enzyme–OP species. Since MEPQ is a racemic compound, it produced equal amounts of two enzyme–OP species that exhibited different rates of reactivation in the presence of oximes. According to Ashani *et al.* [3], the fast-reactivation species is the one that is formed by the Sp enantiomer of MEPQ with enzyme, while the slow-reactivation species is the one formed by the Rp enantiomer. Our studies using Sp iPrMP and SpSc soman support this contention, since considerably fast rates of reactivation were observed for AChE inhibited by these two bulkier Sp enantiomers. It is also interesting to note that increasing the size of the alkoxy group from ethyl or isopropyl to pinacolyl only slightly reduced the rate of reactivation by HI-6. This indicates that steric hindrance caused by the SpSc soman moiety does not play a significant role in HI-6-induced reactivation. The W86 located at the choline-binding site may be used by HI-6 in orienting the oxime to attack the OP moiety. However, Sp iPrMP- and SpSc soman-inhibited W86A AChE promoted HI-6 reactivation at a rate comparable to that of wild-type enzyme, indicating that HI-6 does not need the facilitation of choline-binding site W86 for the nucleophilic attack by the oxime molecule.

W286 is regarded as the core component of the peripheral anionic site of AChE and only moderate reductions in the second-order rate constants were demonstrated in HI-6-induced reactivations of W286A AChE conjugated with all Sp methylphosphonates. However, another periperal site mutant, Y124Q, produced 36- to 155-fold reductions in the reactivation rate constants with these three methylphosphonates, indicating that Y124 is the residue at the peripheral site that plays an important role in facilitating HI-6-induced reactivation of the Sp methylphosphonate–enzyme conjugates.

The 5- and 3-fold lower second-order reactivation rate constants of paraoxon-inhibited AChE compared to those of Sp iPrMP- and SpSc soman-inhibited AChE, respectively, are difficult to reconcile on the basis of steric rationale that AChE inhibited by a small organophosphate, paraoxon, is more refractory to HI-6 reactivation than that inhibited by the bulkier methylphosphonates. Since 2-PAM, TMB4, and obidoxime are more efficient reactivators of diethylphosphorylated AChE than HI-6 [5,14,15], it is likely that a factor, other than steric hindrance, is responsible for the refractoriness of this conjugate towards HI-6 reactivation. Molecular modeling studies suggest that the orientation of the OP moiety in the active center is crucial for achieving fast oxime-induced reactivation of AChE conjugates with the Sp enantiomers, but not with the Rp enantiomers of methylphosphonates [5]. Since the structures of both MEPQ and paraoxon share a common ethoxy group, if the other ethoxy group of paraoxon can assume an orientation in the acyl pocket, the reactivation of paraxon-inhibited mutant enzymes should follow a pattern similar to that of AChE inhibited by the Sp enantiomer of MEPQ. However, the reactivation study with HI-6 showed that the pattern of the reactivation of different mutant enzymes inhibited with paraoxon is different from all Sp methylphosphonate-inhibited enzymes. This indicates that the slower HI-6 reactivation rate observed with paraoxon-inhibited enzyme is due to a different orientation of the OP residue in the active site gorge compared with the Sp methylphosphonates. This is also supported by the study with mutant Y124Q, which demonstrated $\geq$36-fold decrease in the reactivation rate constants with all Sp methylphosphonate–enzyme conjugates. Reactivation of paraoxon-inhibited Y124Q resulted in a 6-fold decrease in the second-order rate constant, suggesting that the peripheral site of the enzyme is not as important in the reactivation of paraoxon-inhibited AChE as it is in the reactivation of methylphosphonate–enzyme conjugates. At the same time, the choline-binding site mutant, W86A, reduced the reactivation rate constant of paraoxon-inhibited enzyme much more significantly than it did with all the Sp methylphosphonates. This indicates that, unlike the reactivation of Sp methylphosphonate-inhibited AChEs, HI-6 may use the choline-binding site to orient the oxime molecule for nucleophilic attack in the reactivation of paraoxon-inhibited AChE. Therefore, the results of site-directed mutagenesis studies suggest that HI-6 may take two different orientations in the reactivation of AChE inhibited by the Sp methylphosphonates and organophosphate, paraoxon.

Reactivation of wild-type AChE by HI-6 analog showed that deletion of the ether oxygen only reduced the reactivation potency for the fast-reactivation species of MEPQ-inhibited enzyme, which is the conjugate formed by the Sp enatiomer of MEPQ. For the slow-reactivation species and paraoxon-inhibited enzyme, deletion of the ether oxygen had negligible effect on the reactivation potency of the oxime. This also supports the hypothesis that HI-6 assumes different orientations during the reactivation of AChE inhibited by organophosphate paraoxon and Sp methylphosphonates.

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