### Title

Catalytic Soman Scavenging by the Y337A/F338A Acetylcholinesterase Mutant Assisted with Novel Site-Directed Aldoximes




### Journal

Chemical Research in Toxicology, 28(5)

### ISSN

0893-228X



### Authors

Kovarik, Zrinka
Hrvat, Nikolina Maček
Katalinić, Maja
et al.



### Publication Date

2015-05-18

### DOI

10.1021/acs.chemrestox.5b00060

Peer reviewed

HHS Public Access

Author manuscript

*Chem Res Toxicol*. Author manuscript; available in PMC 2016 March 14.

Published in final edited form as:

*Chem Res Toxicol*. 2015 May 18; 28(5): 1036–1044. doi:10.1021/acs.chemrestox.5b00060.

# Catalytic soman scavenging by Y337A/F338A acetylcholinesterase mutant assisted with novel site-directed aldoximes

**Zrinka Kovarik**[†,*], **Nikolina Maček Hrvat**[†], **Maja Katalinić**[†], **Rakesh K. Sit**[‡], **Alexander Paradyse**[§], **Suzana Žunec**[†], **Kamil Musilek**[||], **Valery V. Fokin**[‡], **Palmer Taylor**[§], and **Zoran Radić**[§,*]

[†]Institute for Medical Research and Occupational Health, Ksaverska cesta 2, HR-10000 Zagreb, Croatia

[‡]Skaggs Institute for Chemical Biology and Department of Chemistry, The Scripps Research Institute, La Jolla, CA 92037, USA

[§]Department of Pharmacology, Skaggs School of Pharmacy & Pharmaceutical Sciences, University of California at San Diego, La Jolla, CA 92093-0650, USA

[||]University of Hradec Kralove, Faculty of Science, Department of Chemistry, Rokitanskeho 62, 50003 Hradec Kralove, Czech Republic

## Abstract

Exposure to the nerve agent soman is difficult to treat due to the rapid dealkylation of soman-acetylcholinesterase (AChE) conjugate known as aging. Oxime antidotes commonly used to reactivate organophosphate inhibited AChE are ineffective against soman, while the efficacy of the recommended nerve agent bioscavenger butyrylcholinesterase is limited by strictly stoichiometric scavenging. To overcome this limitation, we tested *ex vivo*, in human blood, and *in vivo*, in soman exposed mice, the capacity of aging-resistant human AChE mutant Y337A/F338A in combination with oxime HI-6 to act as a catalytic bioscavenger of soman. HI-6 was previously shown *in vitro* to efficiently reactivate this mutant upon soman, as well as VX, cyclosarin, sarin and paraoxon inhibition. We here demonstrate that *ex vivo*, in whole human blood, 1 μM soman was detoxified within 30 minutes when supplemented with 0.5 μM Y337A/F338A AChE and 100 μM HI-6. This combination was further tested *in vivo*. Catalytic scavenging of soman in mice improved the therapeutic outcome and resulted in the delayed onset of toxicity symptoms. Furthermore, in a preliminary *in vitro* screen we identified an even more efficacious oxime than HI-6, in a series of forty-two pyridinium aldoximes, and five imidazole 2-aldoxime N-propyl pyridinium derivatives. One of the later imidazole aldoximes, RS-170B, was a 2–3 –fold more

[*]Corresponding Author: Zrinka Kovarik, Institute for Medical Research and Occupational Health, Ksaverska cesta 2, P. O. Box 291, HR-10001 Zagreb, Croatia, zkovarik@imi.hr. Zoran Radić, Skaggs School of Pharmacy & Pharmaceutical Sciences 0650, University of California at San Diego, La Jolla, CA 92093, USA, zradic@ucsd.edu.

**Author Contributions**

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Supporting Information. This article contains supplemental synthetic details with Schemes 1 and 2, Table S1 and Figures S1–S4. This material is available free of charge via the Internet at http://pubs.acs.org.

effective reactivator of Y337A/F338A AChE than HI-6 due to the smaller imidazole ring, as indicated by computational molecular models, that affords a more productive angle of nucleophilic attack.

**Keywords**

organophosphate antidotes; oxime cholinesterase reactivators; soman scavenging; acetylcholinesterase reactivation

## Introduction

Organophosphorus compounds (OP), such as the nerve agents, soman, tabun, sarin and VX, used in terrorism and chemical warfare, are extremely potent inhibitors of acetylcholinesterase (AChE, EC 3.1.1.7). Their toxicity is mainly based on their ability to phosphylate serine of the AChE catalytic triad, consequently causing the accumulation of the neurotransmitter acetylcholine (ACh) and resulting in toxicity leading to death. In the event of OP poisoning, immediate therapeutic treatment includes the use of an anticholinergic drug such as atropine to antagonize the effects of ACh accumulation by binding to the muscarinic ACh receptors, an anticonvulsant and an oxime as a reactivator of inhibited AChE. Among nerve agents, soman is uniquely difficult to counteract because soman-inhibited AChE quickly becomes unresponsive to reactivation due to the rapid dealkylation (known as aging) of the soman-AChE conjugate with a half time of only 2 min.[1,2] The anionic methylphosphonylated-AChE conjugate formed by aging is no longer susceptible to oxime reactivation, in part due to the charge repulsion between the anionic oximate and the conjugated methylphosphonic acid.[3] Therefore, a novel approach to treat soman exposure is a necessity.

Current investigations are focused on administrating human enzymes or plasma capable of inactivating OP compounds before they react with the target AChE.[4–8] These enzymes are either stoichiometric bioscavengers, such as plasma butyrylcholinesterase (BChE; E.C. 3.1.1.8), that covalently bind OPs,[4] pseudo-catalytic, engineered AChE mutants capable of OP degradation when assisted by oximes[9–11] or other phosphoesterases that catalyze hydrolysis of OP compounds.[12–16] Recently, several mutants of human AChE were created with the aim of slowing the aging rate and providing increased oxime accessibility to the phosphylated catalytic serine.[17] An initial screening of a library of 840 oximes for reactivation of the soman inhibited human AChE mutant Y337A/F338A opened new avenues in scavenging and antidotal research, showing oxime-enhanced reactivation. However, no oxime in the *in vitro* tests surpassed the standard bispyridinium oxime HI-6 (Cochran et al. 2011).

In the present study we evaluated *in vitro*, *ex vivo* and *in vivo* bioscavenging potential of the combination comprising the Y337A/F338A mutant and HI-6 in order to prove the principle of oxime-assisted catalytic scavenging of soman. Simultaneously, we continued reiterative synthetic and efficacy oriented search for more effective reactivators by an analysis of a library of HI-6 analogs. We identified a reactivator of the soman-inhibited mutant superior to HI-6 thus establishing a base to improve oxime-assisted catalytic scavengers for soman.

## Materials and Methods

### Chemicals

Oximes RS-163B, RS-167B and RS-169A were prepared and characterized as described in the Supplemental materials. Oximes RS-170B and RS-95C were prepared as described earlier.[18] K-oximes, HS 6, TMB-4, 4-PAM, and obidoxime were obtained from the Faculty of Military Health Sciences, Hradec Králové, Czech Republic, while ICD585, DMB-4, MMB-4, and HLö7 from the US Army Medical Research Institute of Chemical Defense, Aberdeen Proving Ground, MD, USA. HI-6 was purchased from US Biological (Swampscott, MA). Chemical structures of tested oximes are shown in Fig. S1. Oxime stock solutions were prepared in water or buffer and diluted just before use.

Organophosphate soman [*O*-pinacolyl-methyl-phosphonofluoridate] was purchased from NC Laboratory, Spiez, Switzerland. Soman was diluted in isopropyl alcohol and further dilutions in water were made before use. The non-volatile, low toxicity soman analogue, pinacolyl 3-cyano-4-methyl-7-hydroxy coumarin methyl phosphonate[19] was used to prepare the soman–hAChE conjugate, identical with the one formed upon inhibition with soman, for *in vitro* reactivation by imidazole-pyridinium oximes and HI-6.

Acetylthiocholine iodide (ATCh) and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) were purchased from Sigma Chemical Co., St. Louis, MO, USA. Final concentrations of ATCh and DTNB were 1.0 mM and 0.3 mM, respectively.

### Enzymes

Recombinant human AChE wild type and human AChE mutant Y337A/F338A were prepared as described earlier.[17] Human whole blood (hWB) was collected at the Institute for Medical Research and Occupational Health, Zagreb, Croatia. Enzyme activity was measured by the Ellman method.[20]

### In vitro enzyme activity assays

Progressive inhibition of wild-type human AChE and its mutant Y337A/F338A by soman was measured after a given time of enzyme incubation (up to 30 min) with soman (0.5–10 nM). The second-order rate constant of inhibition by soman ($k_i$) was calculated as described previously.[21]

The aging rate constant of soman-inhibited Y337A/F338A, $k_a$, was determined as follows: after a 4 min incubation of mutant with ten-fold excess soman concentration to achieve 95–100 % inhibition, the excess of unconjugated soman was rapidly removed by column filtration (Sephadex G-50 spin column, Roche Diagnostic GmbH, Mannheim, Germany). The reactivatable mutant fraction in time was determined by measuring enzyme activity after 10 min incubation with 1 mM HI-6. Activity was routinely corrected for the inhibitory effect of HI-6 and for HI-6-induced hydrolysis of ATCh. The first-order rate constant of aging, $k_a$, was determined as a slope from the linear regression of logarithm of the reactivatable fraction against time.

For the *ex vivo* assay of soman detoxification, hWB was supplemented with 0.5 μM Y337A/F338A mutant and incubated with soman in concentration range from 0.5 to 10 μM. After 10 to 15 min of inhibition (achieving 95–100 % inhibition), the oxime (0.10 or 1.0 mM) was added to the mixture. At specified time intervals, an aliquot was diluted for enzyme activity measurements, expressed as percentage of control activity. Reactivation screening was performed at a single oxime concentration (1.0 mM). The Y337A/F338A mutant was incubated with a ten-fold excess soman or soman analogue concentration for about 2–5 min achieving 95–100 % inhibition. The inhibited enzyme was passed through a Sephadex G-50 spin column (Roche Diagnostic GmbH, Mannheim, Germany) to remove an excess of unconjugated soman. Then, the enzyme was incubated with an oxime, and at specified time intervals, up to 22 h, an aliquot was diluted for enzyme activity measurement. An equivalent sample of uninhibited enzyme was passed through a parallel column, diluted to the same extent as the inhibition mixture, and control activity was measured in the presence of oxime. Both activities of control and reactivation mixture were corrected for oxime-induced hydrolysis of ATCh. The observed first-order reactivation rate constant ($k_{obs}$) and maximal percentage of reactivation ($React_{max}$) were calculated as described previously.[22] Detailed reactivation kinetics for leading oximes in concentration range from 0.10 up to 3.0 mM, was analyzed to determine the maximal reactivation rate constant, $k_2$, phosphonylated enzyme-oxime dissociation constant, $K_{OX}$, and the overall second-order reactivation rate constant, $k_r$, as described previously.[23]

The enzyme-oxime dissociation constant $K_i$ was determined for selected leading oximes from the effect of ATCh concentration (50–500 μM) on the degree of reversible inhibition by oximes (50–300 μM) as described previously.[24]

All activity measurements were performed in 0.1 M sodium phosphate buffer containing 0.01 % BSA, pH 7.4, at 25 °C and 412 nm (or 436 nm), on a CARY 300 spectrophotometer (Varian Inc., Australia) with a temperature controller. Reversible inhibition was measured on a plate reader Infinite M200PRO (Tecan, Switzerland).

## Detoxification of soman-exposed mice by combining human AChE mutant Y337A/F338A with HI-6

Male CD-1 mice of 25–30 g body weight (purchased from Ru er Bošković Institute, Zagreb, Croatia) were fed on a standard diet; they had free access to water and were kept in Macrolone cages at 21 °C, exchanging light and dark cycles every 12 h. Mice were divided into groups of four for each dose. Acute toxicity ($LD_{50}$) was based upon 24 h mortality rates and calculated according to Thompson[25] and Weil[26]. Antidotal efficacy of HI-6 therapy alone against soman poisoning was tested by administering HI-6 intramuscularly to mice (110 mg/kg, a dose equal to 25 % of HI-6 $LD_{50}$ where no toxic signs were observed) together with atropine sulfate (10 mg/kg) one minute after subcutaneous soman exposure as described previously.[27] To test the catalytic detoxification of soman-exposed mice *in vivo*, a combination of pretreatment and therapy was applied.[28,29] Mice were pretreated intravenously with HI-6 (70 or 110 mg/kg) and Y337A/F338A (1.0 mg/kg) 5 or 15 min prior to soman exposure and then treated by HI-6 (110 mg/kg) in atropine (10 mg/kg). The antidotal efficacy of treatments was expressed as a protective index (PI) with 95 %

confidence limits and maximal dose of poison (MDP). The PI was the ratio of $LD_{50}$ between soman with antidote treatments and soman given alone. The MDP was the maximal dose of the soman $LD_{50}$ that was fully counteracted by the treatment applied. The mice were treated in accordance with the approval of the Ethics Committee of the Institute for Medical Research and Occupational Health in Zagreb, Croatia.

### Computational molecular modeling

Models of oximes RS-170B, HI-6 and RS-169A were prepared with Insight II (Accelrys, San Diego) as described earlier for HI-6 and similar imidazole oximes.[17,18] The crystal structure of soman-TcAChE conjugate (PDB id 2WFZ) was used as a source of phosphonylated active center serine, and was pasted into 3D structure of human AChE (PDB id 4EY4). All water molecules and reversibly bound ligands were removed, while incompletely resolved amino acid side-chains were repaired. Oximes were oriented into the soman-hAChE gorge with their oximate oxygens approx. 4 Å from the conjugated phosphorus atom. A flexible distance constraint was placed between those two atoms at 3 Å, and molecular dynamics (MD) calculations were performed as described before.[18] Only selected hAChE side-chains (Y70, Y124, S203, W286, F295, F297, Y341 and H447) and the phosphonyl conjugate were allowed to rotate during simulation together with oxime. Ten calculations were performed per oxime. Resulting structures were visualized using Discovery Studio Visualizer 4.0 (Accelrys, San Diego).

## Results

Soman detoxification assisted by combining human AChE mutant Y337A/F338A with HI-6. In present study we characterized interactions of soman and the double mutant determining the inhibition constant by soman, the aging rate of soman-inhibited mutant, as well as reversible inhibition constant for HI-6 (Table 1). The soman-mutant conjugate had 26-fold slower aging rate in comparison to the wild type human AChE and its halftime of aging was 50 min (*vs.* 2 min for the wild-type human AChE[1,2]). On the other hand, the two introduced mutations did not compromise appreciably AChE phosphonylation with soman. The mutant was only 20% less efficiently inhibited with soman than the wild-type AChE ($k_i$ constants, Table 1), along with a relatively small reduction in binding affinity for HI-6 ($K_i$ constants, Table 1). Therefore, the soman scavenging capacity of the mutant combined with HI-6 appeared rate limited primarily on the efficiency of HI-6 to dephosphorylate AChE and recover the mutant activity through continuous turnover. The scavenging capacity was tested *ex vivo* at four concentrations of soman selected to mimic concentrations likely to be found in the blood of an organophosphate-exposed person.[30] The mutant AChE concentration was close to an equivalent dose of human plasma BChE (0.5–2.5 mg/kg *i.v.)* previously tested for bioscavenging,[29] but still about ten-fold lower than the dose recommended for administration of BChE as stoichiometric OP bioscavenger in the treatment of an OP exposure *in vivo*.[31] Soman detoxification monitored in hWB supplemented with human AChE mutant Y337A/F338A showed significant soman detoxification and consequential recovery of mutant activity following addition of HI-6 (Fig. 1). No reactivation or detoxification of soman was observed if the mutant AChE and oxime were not present in hWB. Therefore, total recovered activity denotes reactivation of the mutant, since mutant

activity contributed to approximately 70 % of control activity (hWB supplemented with mutant). The most rapid soman detoxification was observed within the initial twenty minutes of oxime-assisted catalysis, when hWB was supplemented with 0.5 μM Y337A/F338A and 1.0 mM HI-6. The initial recovery of activity with higher soman concentrations had a lag phase preceding the recovery (Fig. 1). Accordingly, soman was degraded by cycles of Y337A/F338A re-inhibition and reactivation until the rate of inhibition was lower than the rate of reactivation, and then the catalytic activity of the mutant increased to its maximum as a result of total soman detoxification by the mutant and its reactivator. The observed multiphasic kinetics could also be due, in part, to differences in inhibition and reactivation kinetics of the four soman diastereoisomers[32,33] leading to different rates of their catalytic hydrolysis and departures from the initial concentration ratio of the racemic mixture.

## Detoxification of soman-exposed mice

Catalytic scavenging of the mutant combined with HI-6 to degrade soman was assayed *in vivo* in mice. The acute toxicity of HI-6 for mice was 450 mg/kg for *i.m.* and 280 mg/kg for *i.v.* application, classifying HI-6 as a reactivator of lower toxicity and confirming our previous results.[27] No toxicity symptoms were observed upon *i.v.* administration of human AChE mutant Y337A/F338A (1 mg/kg). The antidotal efficacy of tested scavenging system in terms of protective index (PI) and maximal dose of soman (MDP) is given in Table 2. The post-exposure therapy with HI-6 alone ensured four-fold protection of mice, while pretreatment of mice with HI-6 additionally improved the antidotal efficacy by 35 %. Furthermore, the 5-min HI-6 plus mutant combined pretreatment and a higher dose of HI-6 in therapy increased the protective index by 60 % compared to the protection observed with only HI-6 therapy. Although the maximal dose of soman (MDP) survived by all of the mice was not affected (5.0 x $LD_{50}$ of soman) regardless of whether the mice received the mutant in pretreatment, the symptoms of toxicity in mice pretreated with a combination of mutant and HI-6 were significantly less intense; e.g. tremor, convulsions, breathing, and locomotion disturbances (Table 3, Table S1). Moreover, even at the highest dose of soman (10 x $LD_{50}$), a delay of 30 min in the time of lethality was noticeable if mice were pretreated with the mutant plus HI-6 combination (Fig. 2).

## Screening of the HI-6 analog soman-Y337A/F338A reactivator library

Newly synthesized pyridinium-linked imidazole 2-aldoximes as well as previously synthesized linked bispyridinium analogs, containing an aldoxime on one or both rings, were screened for their ability to reactivate soman inhibited mutant. Results were sorted in terms of the observed first-order reactivation rate constant ($k_{obs}$) as shown in Fig. 3. Pyridinium groups linked to the imidazole aldoximes were generally more efficient reactivators. RS-170B was 2.5-fold faster in reactivation of soman-inhibited mutant than HI-6 which was followed by RS-163B and RS-95C. Shifting of the position of the carboxylamide substitution in the pyridinium ring or its removal reduced the reactivation rate of the RS-163B and RS-65C analogs of RS-170B up to 5.5.-fold compared to RS-170B or up to 2-fold when compared to HI-6. Bispyridinium oximes HS 6 and ICD-585 followed the downward trend with up to 5.5-fold reduced reactivation rate (compared to HI-6). However, none of the tested oximes restored the soman-inhibited mutant activity to

completion. Out of 47 tested oximes, 21 restored more than 30 % of soman-inhibited mutant activity and the highest maximal percentage of reactivation, between 50 and 70 %, was obtained by 12 oximes among which were 9 bispyridinium oximes with the *ortho*-positioned oxime group, five of which had a $CH_2$-O-$CH_2$ linker.

### Reactivation of soman-Y337A/F338A conjugate by leading oximes

The preferences observed in the screening as well as our previously determined constants[17] for HI-6 were confirmed when selected oximes were tested in wide range of concentrations (0.10–3.0 mM). Evaluation of individual reactivation constants $k_{+2}$ and $K_{OX}$ (Table 4, Fig. 4) revealed that variability in the overall oxime reactivation refers largely (for three out of five oximes) to its interaction with the phosphonate group of the soman conjugated enzyme through forming the transition state of reactivation that is reflected in about 10-fold difference in $k_{+2}$. Covalent soman conjugation affected binding affinity ($K_{OX}$ *vs.* $K_i$), lowering the affinity of the oxime for the soman-Y337A/F338A conjugate (up to 6-fold for HI-6).

### Soman detoxification assisted by human AChE mutant Y337A/F338A and oxime RS-170B

The catalytic scavenging by mutant and RS-170B was tested *ex vivo*. Upon addition of 1 mM RS-170B to hWB, supplemented with 0.5 μM Y337A/F338A previously inhibited by 1.0 μM soman, activity was restored up to 60 % within 5 min (Fig. 5) indicating efficient catalyzed soman detoxification. There was virtually no lag phase preceding the activity recovery by higher RS-170B concentrations when AChE was inhibited by higher (5 μM and 10 μM) soman concentrations (Fig. 5), as was the case of HI-6 (Fig. 1) or other oximes (Fig. S2).

### Computational molecular modeling

Simulation of the interaction of the lead soman-Y337A/F338A conjugate reactivators RS-170B and HI-6 compared to ineffective reactivator RS-169A, supported the experimental data by revealing that the highest frequency of reaction suitable conformations are formed by RS-170B, followed by HI-6 and to significantly smaller extent by bisquaternary RS-169A (Fig. 6 and Fig. S3). It appears that reduced size of the uncharged imidazole 2-aldoxime ring system facilitates tertiary imidazole (but not the quaternary methyl-imidazolium) 2-aldoxime to approach conjugated phosphorus atom with smallest deviations from a preferred angle for an in line SN2, nucleophilic reactivation attack within the spatially impacted organophosphate-conjugated active center gorge. (Fig. 7; Fig. S4).

## Discussion

The investigation presented here was guided by the hypothesis that the administration of a mixture of the aging-resistant human AChE mutant Y337A/F338A and an oxime as a reactivator could provide considerable improvement in soman exposure treatment creating a unique scavenging system. Promising reactivation results[17] and the low acute toxicity of HI-6 for mice[27] allowed us to test this oxime-assisted catalytic bioscavenger system *in vivo* in soman-exposed mice. Using a varied administration regimen of HI-6 and mutant AChE (pretreatment/post-exposure therapy), we observed a significant delay in symptoms of

poisoning and time of death, although the maximal dose of soman was unaffected compared to when only HI-6 was administered to soman-exposed mice as pretreatment. This proved that HI-6 antidotal regimens are restricted to the peripheral tissues as well as its short circulation time (biological half-life was 11 min).[5,34] Nevertheless, we assume that attenuated symptoms such as tremor, breathing, and locomotor disturbances in mice pretreated with mutant and HI-6 reflected the catalytic turnover of soman by the mutant plus HI-6 combination acting as an oxime-assisted catalytic bioscavenger *in vivo*. Our data are the first to establish an *in vivo* example of effective oxime-assisted catalytic soman bioscavenging based on combined administration of sub-stoichiometric amounts of site-directed mutant AChE and an oxime reactivator. In situations of exposure to multiple organophosphates, the oxime could assist scavenging in the blood as well serve as a conventional antidote in target tissues.

Screening of the slow-aging Y337A/F338A AChE mutant with 47 pyridinium-linked imidazole, methylimidazolium, and pyridinium aldoximes, (Fig. S1) revealed that the newly synthesized RS-170B oxime is superior to HI-6 as a reactivator of soman-inhibited Y337A/F338A. We also confirmed previous studies that revealed that the aldoxime group in the *ortho*-position to the pyridinium nitrogen is important structural feature for potent reactivation of soman-inhibited AChE.[17,27,35,36] Our computational calculations indicated preferential facilitated access of smaller, uncharged tertiary imidazole 2-aldoxime-bearing moiety compared to pyridinium or methyl-imidazolium in accessing soman-conjugated phosphorus of soman in Y337A/F338A AChE. Our findings pointed out that oxime-assisted scavenging relies on rapid reactivation to facilitate turnover of the parent organophosphate and a suitable oxime affinity to bind to the soman-phosphonylated mutant. Moreover, the Y337A/F338A mutant reacted with soman as efficiently as the wild-type, establishing that scavenging capacity is limited only by the efficiency of oxime to regenerate active mutant AChE through continuous turnover. In general, kinetic requirements for optimal scavenging necessitate high rates of phosphylation as well as reactivation. A verification of the scavenging potential of the mutant plus reactivator was obtained with *ex vivo* assays in which soman at low micromolar concentrations, that are expected to accumulate in the blood of exposed individuals, was degraded appreciably within 5 to 40 minutes after RS-170B or HI-6 administration into the hWB supplemented with mutant AChE.

In summary, our study has demonstrated through a combination of *in vitro*, *in silico*, *ex vivo*, and *in vivo* approaches, a feasible approach to the development of an oxime-assisted catalytic bioscavenger of soman based on an aging-resistant human AChE mutant in combination with its efficient reactivator. Further bioscavenging developments should consider not only optimization of mutant/oxime doses applied but also improvements such as slowing oxime clearance in blood, which would enable extending pretreatment times and increasing efficiency of scavenging in the plasma before an organophosphate distributes and/or crosses the blood-brain barrier. One of the ways for the mutant AChE to gain prolonged circulation time is a conjugation with polyethylene glycol.[16,37] Such an enzyme should retain all of the kinetic characteristics of its nonPEGylated form while having 20-fold greater mean residence time.[37] Further analysis of soman scavenging should also be directed to stereoselectivity. Soman has four optical isomers consisting of two pairs of diastereo-

isomers having two chiral centers: one on the phosphorus atom (P) and a second on the asymmetric carbon (C) atom of the pinacolyl group [P(−)C(+), P(−)C(−), P(+)C(+) and P(+)C(−) stereo-isomers]. Benschop et al. have found that the pair of soman diastereo-isomers configured with the (−) symmetry on the P atom are 20–150-fold more toxic than the P(+)C(+/−) pair of diastereo-isomers.[33,38] One advantage of the cholinesterases as bioscavengers is that stereoselective preference for the $P_S$ over the $P_R$ enantiomer for inactivation likely matches the preference for reactivation, as we have shown previously for the wild type AChE and a series of methylphosphonates.[23,39] Hence the more toxic enantiomer formed with excess organophosphate is also most susceptible to reactivation.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

We thank Ms Jasna Mileković for assistance with *in vivo* experiments. This work was supported by the NIH (U01 NS058046 and R21NS072086), Croatian Ministry of Science, Education and Sports Grant (022-0222148-2889), Croatian Science Foundation (4307) and University of Hradec Kralove (long-term development plan).

## Abbreviations

| | |
|---|---|
| **ACh** | acetylcholine |
| **AChE** | acetylcholinesterase |
| **ATCh** | acetylthiocholine iodide |
| **BChE** | butyrylcholinesterase |
| **DTNB** | 5,5′-dithiobis-2-nitrobenzoic acid |
| **hAChE** | human acetylcholinesterase |
| **hWB** | human whole blood |
| **MD** | molecular dynamics |
| **MDP** | maximal dose of poison |
| **OP** | organophosphorus compound |
| **PI** | protective index |
| **TcAChE** | *Torpedo califonica* acetylcholinesterase |

## References

1. De Jong LPA, Wolring GZ. Stereospecific reactivation by some Hagedorn-oximes of acetylcholinesterases from various species including man, inhibited by soman. Biochem Pharmacol. 1984; 33:1119–1125. [PubMed: 6712722]
2. Shafferman A, Ordentlich A, Barak D, Stein D, Aiel N, Velan B. Aging of phosphylated human acetylcholinesterase: catalytic processes mediated by aromatic and polar residues of the active centre. Biochem J. 1996; 318:833–840. [PubMed: 8836126]

3. Barak D, Ordentlich A, Segall Y, Velan B, Benschop HP, De Jong LPA, Shafferman A. Carbocation-mediated processes in biocatalysts: contribution of aromatic moieties. J Am Chem Soc. 1997; 119:3157–3158.
4. Ashani Y, Shapira S, Levy D, Wolfe AD, Doctor BP, Raveh L. Butyrylcholinesterase and acetylcholinesterase prophylaxis against soman poisoning in mice. Biochem Pharmacol. 1991; 41:37–41. [PubMed: 1986743]
5. Maxwell DM, Brecht KM, Doctor BP, Wolfe AD. Comparison of antidote protection against soman by pyridostigmine, HI-6 and acetylcholinesterase. J Pharmacol Exp Ther. 1993; 264:1085–1089. [PubMed: 8450452]
6. Lenz DE, Yeung D, Smith RJ, Sweeney RE, Lumley LA, Cerasoli DM. Stoichiometric and catalytic scavengers as protection against nerve agent toxicity: A mini review. Toxicology. 2007; 233:31–39. [PubMed: 17188793]
7. Vučinić S, Zlatković M, Antonijević B, Ćurčić M, Bošković B. Fresh frozen plasma as a successful antidotal supplement in acute organophosphate poisoning. Arh Hig Rada Toksikol. 2013; 64:273–277.
8. Wille T, Thiermann H, Worek F. *In vitro* kinetics of nerve agent degradation by fresh frozen plasma (FFP). Arch Toxicol. 2014; 88:301–307. [PubMed: 24057572]
9. Kovarik Z, Radić Z, Berman HA, Taylor P. Mutation of acetylcholinesterase to enhance oxime-assisted catalytic turnover of methylphosphonates. Toxicology. 2007; 233:79–84. [PubMed: 17046138]
10. Taylor P, Reiner E, Kovarik Z, Radić Z. Application of recombinant DNA methods for production of cholinesterases as organophosphate antidotes and detectors. Arh Hig Rada Toksikol. 2007; 58:339–345. [PubMed: 17913689]
11. Nachon F, Brazzolotto X, Trovaslet M, Masson P. Progress in the development of enzyme-based nerve agent bioscavengers. Chem Biol Interact. 2013; 206:536–544. [PubMed: 23811386]
12. Harel M, Aharoni A, Gaidukov L, Brumshtein B, Khersonsky O, Meged R, Dvir H, Ravelli RB, McCarthy A, Toker L, Silman I, Sussman JL, Tawfik DS. Structure and evolution of the serum paraoxonase family of detoxifying and anti-atherosclerotic enzymes. Nat Struct Mol Biol. 2004; 11:412–419. [PubMed: 15098021]
13. Amitai G, Gaidukov L, Adani R, Yishay S, Yacov G, Kushnir M, Teitlboim S, Lindenbaum M, Bel P, Khersonsky O, Tawfik DS, Meshulam H. Enhanced stereoselective hydrolysis of toxic organophosphates by directly evolved variants of mammalian serum paraoxonase. FEBS J. 2006; 273:1906–1919. [PubMed: 16640555]
14. diTargiani RC, Chandrasekaran L, Belinskaya T, Saxena A. In search of a catalytic bioscavenger for the prophylaxis of nerve agent toxicity. Chem Biol Interact. 2010; 187:349–354. [PubMed: 20176006]
15. Goldsmith M, Ashani Y, Simo Y, Ben-David M, Leader H, Silman I, Sussman JL, Tawfik DS. Evolved stereoselective hydrolases for broad-spectrum G-type nerve agent detoxification. Chem Biol. 2012; 19:456–466. [PubMed: 22520752]
16. Melzer M, Heidenreich A, Dorandeu F, Gäb J, Kehe K, Thiermann H, Letzel T, Blum MM. *In vitro* and *in vivo* efficacy of PEGylated diisopropyl fluorophosphatase (DFPase). Drug Test Anal. 2012; 4:262–270. [PubMed: 22174192]
17. Cochran R, Kalisiak J, Küçükkılınç T, Radić Z, Garcia E, Zhang L, Ho YK, Amitai G, Kovarik Z, Fokin VV, Sharpless KB, Taylor P. Oxime-assisted acetylcholinesterase catalytic scavengers of organophosphates that resist aging. J Biol Chem. 2011; 286:29718–29724. [PubMed: 21730071]
18. Sit RK, Fokin VV, Amitai G, Sharpless KB, Taylor P, Radić Z. Imidazole aldoximes effective in assisting butyrylcholinesterase catalysis of organophosphate detoxification. J Med Chem. 2014; 57:1378–1389. [PubMed: 24571195]
19. Amitai G, Adani R, Yacov G, Yishay S, Teitlboim S, Tveria L, Limanovich O, Kushnir M, Meshulam H. Asymmetric fluorogenic organophosphates for the development of active organophosphate hydrolases with reversed stereoselectivity. Toxicology. 2007; 233:187–198. [PubMed: 17129656]

20. Ellman LG, Courtney DK, Andres V, Featherstone MR. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol. 1961; 7:88–95. [PubMed: 13726518]
21. Kovarik Z, Bosak A, Šinko G, Latas T. Exploring the active sites of cholinesterases by inhibition with bambuterol and haloxon. Croat Chem Acta. 2003; 76:63–67.
22. Kovarik Z, Maček N, Sit RK, Radić Z, Fokin VV, Sharpless KB, Taylor P. Centrally acting oximes in reactivation of tabun-phosphoramidated AChE. Chem Biol Interact. 2013; 203:77–80. [PubMed: 22960624]
23. Kovarik Z, Radić Z, Berman HA, Simeon-Rudolf V, Reiner E, Taylor P. Mutant cholinesterases possessing enhanced capacity for reactivation of their phosphonylated conjugates. Biochemistry. 2004; 43:3222–3229. [PubMed: 15023072]
24. Kovarik Z, Čalić M, Šinko G, Bosak A. Structure-activity approach in the reactivation of tabun-phosphorylated human acetylcholinesterase with bispyridinium *para*-oximes. Arh Hig Rada Toksikol. 2007; 58:201–209. [PubMed: 17562604]
25. Thompson WR. Use of moving averages and interpolation to estimate median-effective dose. Bacteriol Rev. 1947; 11:115–145.
26. Weil CS. Tables for convenient calculation of median-effective dose ($LD_{50}$ or $ED_{50}$) and instructions in their use. Biometrics. 1952; 8:249–263.
27. Čalić M, Lucić Vrdoljak A, Radić B, Jelić D, Jun D, Kuča K, Kovarik Z. *In vitro* and *in vivo* evaluation of pyridinium oximes: mode of interaction with acetylcholinesterase, effect on tabun- and soman-poisoned mice and their cytotoxicity. Toxicology. 2006; 219:85–96. [PubMed: 16332406]
28. Berend S, Katalinić M, Lucić Vrdoljak A, Kovarik Z, Kuča K, Radić B. *In vivo* experimental approach to treatment against tabun poisoning. J Enzyme Inhib Med Chem. 2010; 25:531–536. [PubMed: 20235800]
29. Radić Z, Dale T, Kovarik Z, Berend S, Garcia E, Zhang L, Amitai G, Green C, Radić B, Duggan BM, Ajami D, Rebek J, Taylor P. Catalytic detoxification of nerve agent and pesticide organophosphates by butyrylcholinesterase assisted with non-pyridinium oximes. Biochem J. 2013; 450:231–242. [PubMed: 23216060]
30. Munro NB, Ambrose KR, Watson AP. Toxicity of the organophosphate chemical warfare agents GA, GB and VX: implication for public protection. Environ Health Perspect. 1994; 102:18–38. [PubMed: 9719666]
31. Masson P, Lockridge O. Butyrylcholinesterase for protection from organophosphorus poisons: catalytic complexities and hysteretic behavior. Arch Biochem Biophys. 2010; 494:107–144. [PubMed: 20004171]
32. Bucht G, Puu G. Aging and reactivatability of plaice cholinesterase inhibited by soman and its stereoisomers. Biochem Pharmacol. 1984; 33:3573–3577. [PubMed: 6508819]
33. Benschop HP, De Jong LPA. Nerve agent stereoisomers: analysis isolation and toxicology. Acc Chem Res. 1988; 21:368–374.
34. Shih TM, Whalley CE, Valdes JJ. A comparison of cholinergic effects of HI-6 and pralidoxime-2-chloride (2-PAM) in soman poisoning. Toxicol Lett. 1991; 55:131–147. [PubMed: 1998202]
35. Bošković B. The treatment of soman poisoning and its perspectives. Fundam Appl Toxicol. 1981; 1:203–213. [PubMed: 7184784]
36. Schoene K, Steinhanses J, Oldiges H. Reactivation of soman inhibited acetylcholinesterase *in vitro* by bispyridinuim-2-aldoximes. Biochem Pharmacol. 1983; 32:1649–1651. [PubMed: 6860352]
37. Mazor O, Cohen O, Kronman C, Raveh L, Stein D, Ordentlich A, Shafferman A. Aging-resistant organophosphate bioscavenger based on polyethylene glycol-conjugated F338A human acetylcholinesterase. Mol Pharmacol. 2008; 74:755–63. [PubMed: 18523134]
38. Benschop HP, Konings CAG, van Genderen J, De Jong LPA. Isolation, anticholinesterase properties, and acute toxicity in mice of four stereo-isomers of the nerve agent soman. Toxicol Appl Pharmacol. 1984; 72:61–74. [PubMed: 6710485]
39. Kovarik Z, Radić Z, Berman HA, Simeon-Rudolf V, Reiner E, Taylor P. Acetylcholinesterase active centre and gorge conformations analyzed by combinatorial mutations and enantiomeric phosphonates. Biochem J. 2003; 373:33–40. [PubMed: 12665427]

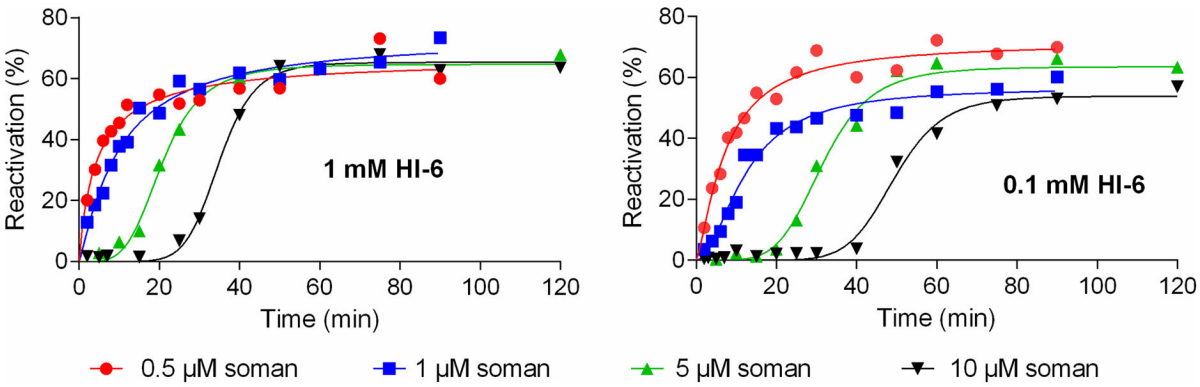


**Figure 1.**

Representative experiment of detoxification of soman in whole human blood supplemented with 0.5 μM human AChE mutant Y337A/F338A and exposed to 0.5 μM-10 μM soman, upon addition of 1 or 0.1 mM HI-6.

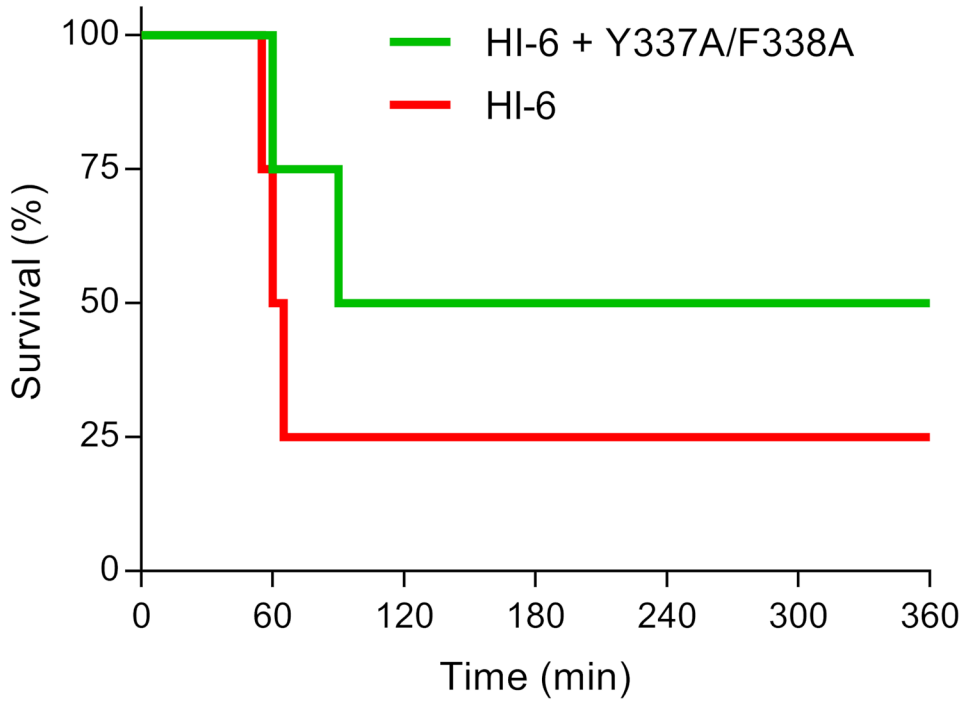


**Figure 2.**

Survival plots for mice pretreated with HI-6 or HI-6 plus human AChE mutant Y337A/F338A 5 min prior to exposure to 10 x $LD_{50}$ of soman. Doses in pre-treatment were 1 mg/kg AChE mutant and 110 mg/kg HI-6. All mice received HI-6 (110 mg/kg) in atropine (10 mg/kg) 1 min after soman exposure. Experiment utilized four mice in each group.

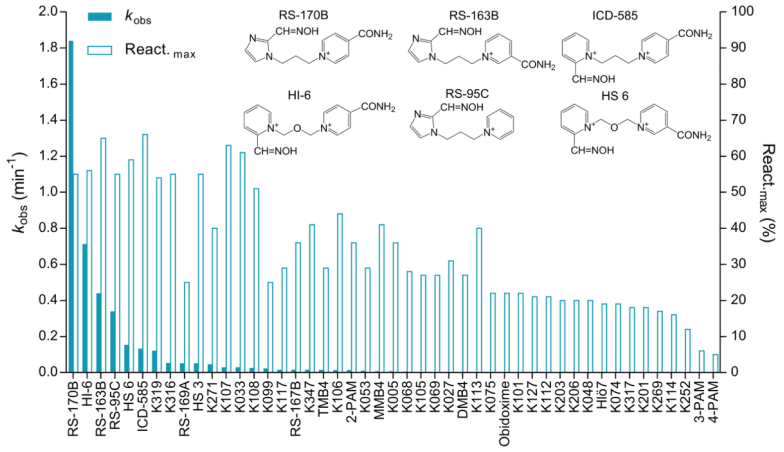


**Figure 3.**

Reactivation of soman-inhibited human AChE mutant Y337A/F338A by 1 mM oximes recorded within 22 h and expressed in terms of the observed first-order reactivation rate constant ($k_{obs}$) and maximal reactivation percentege (React.$_{max}$). A mean of at least three experiments is presented (SD values were less than 10 %, $k_{obs}$ was not calculated when React.$_{max}$ was ≤ 20 %).

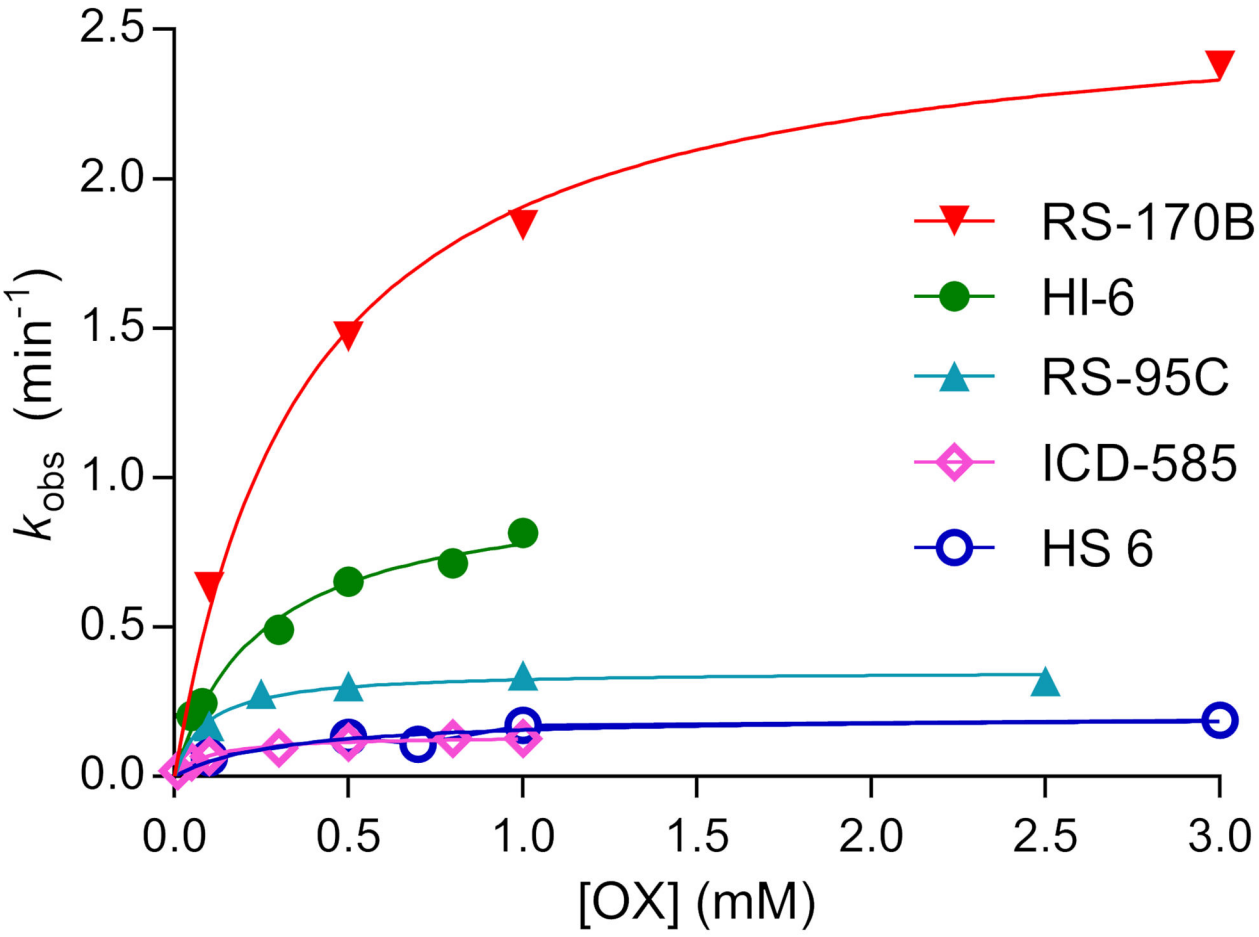


**Figure 4.**

Reactivation kinetics of soman-inhibited human AChE mutant Y337A/F338A by bispyridinium aldoximes (HI-6, ICD-585 and K237) and pyridinium-imidazole aldoximes (RS-170B and RS-95C). Structures are shown in Figure 3. A mean of at least three experiments is presented. Reactivation constants were determined by nonlinear regression of experimental data and are presented in Table 4.

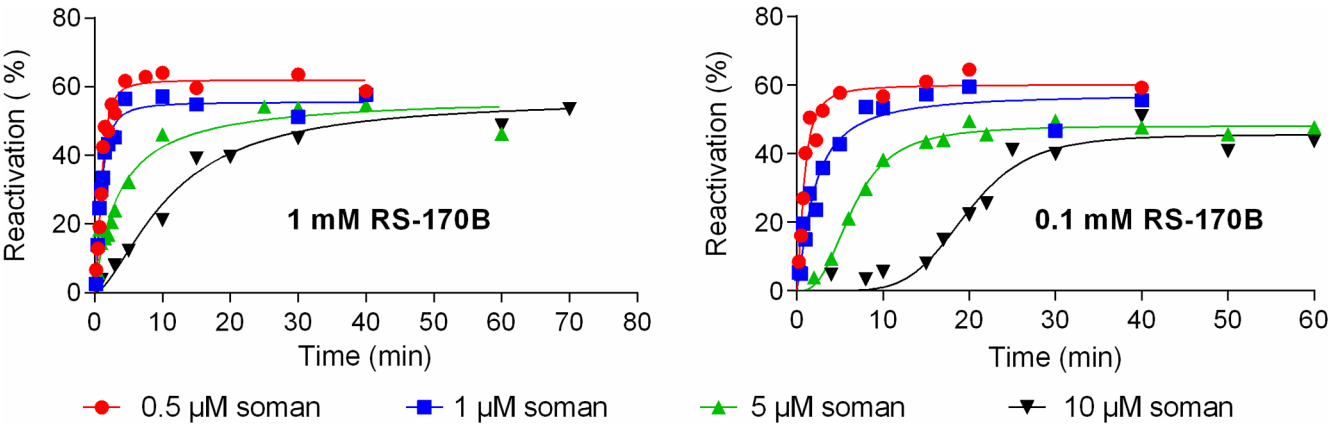


**Figure 5.**

Representative experiment of detoxification of soman in whole human blood supplemented with 0.5 µM human AChE mutant Y337A/F338A and exposed to 0.5 µM-10 µM soman, upon addition of 1 or 0.1 mM RS-170B.

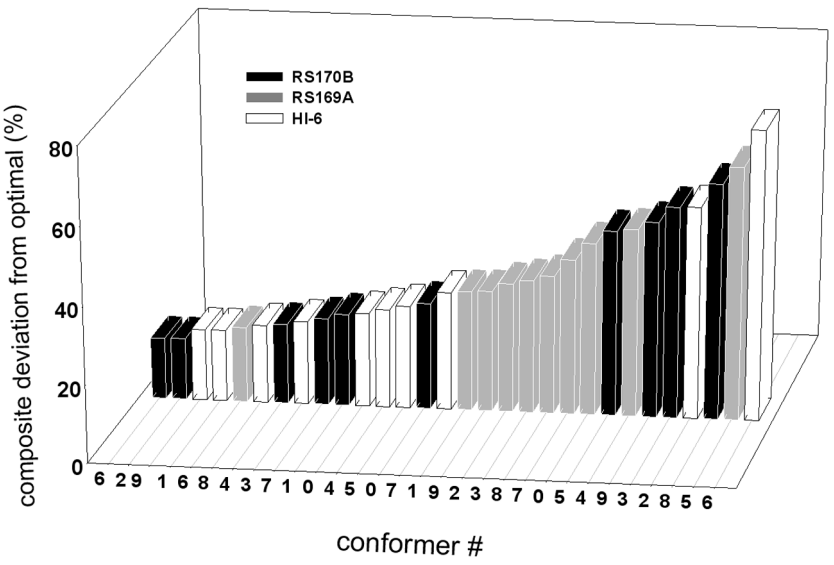


**Figure 6.**

Thirty simulated conformers (cf. Figure S3) of oximes RS-170B (black bars), RS-169A (grey bars) and HI-6 (white bars) rank ordered by individual composite deviations from optimal properties required for reactivation of soman inhibited human AChE mutant Y337A/F338A. Individual composite deviation of each conformer was obtained by averaging percent of deviations (within observed deviation range): a) from the $E_{total}$ of the lowest energy conformer ($\Delta E_{total}$), b) from the minimal observed distance between the nucleophilic oxime O and conjugated phosphorus (-P····O-N=C- distance), c) from the ideal nucleophilic inline attack angle Ser-(O-P····O)-N= (ideally 180°), and from ideal positioning of attacking electron density towards phosphorus atom, Ser-O-(P····O-N)= angle (ideally 109.5°).

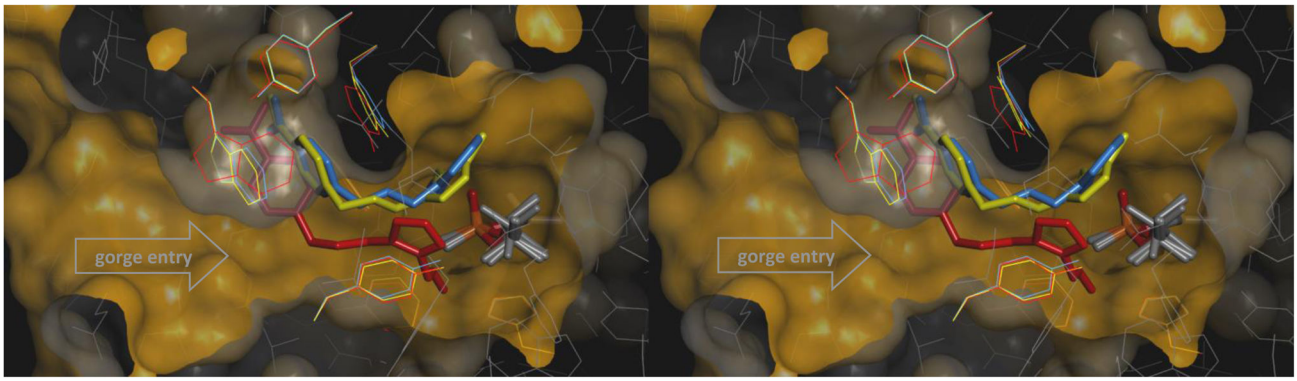


**Figure 7.**

Stereo representation of computational molecular models of oximes RS-170B (red sticks), HI-6 (blue sticks) and RS-169A (yellow sticks) bound reversibly to soman inhibited human AChE mutant Y337A/F338A represented by the orange Connolly surface and side-chains as lines. Side-chains allowed to rotate during simulation were colored after the corresponding oxime, all other side-chains are represented as grey lines. Atoms of phosphonyl Ser203 are represented by sticks, P (orange), C (grey), O (red). The most optimally positioned conformers out of ten calculated conformers (cf. Figures S3 and S4) for the each oxime are shown. A monoquaternary, imidazole aldoxime N-propyl pyridinium, RS-170B binds noticeably deeper in the gorge, as compared to the bisquaternary, imidazolium aldoxime N-propyl pyridinium RS-169A and bisquaternary bispyridinium aldoxime HI-6, while maintaining proper reaction geometry for an SN2 attack on the phosphorus.

**Table 1**

Kinetic constants for inhibition by soman ($k_i$, $\mu M^{-1}$ $min^{-1}$), aging ($k_a$, $min^{-1}$), the overall reactivation rate constant of HI-6-assisted reactivation of soman-enzyme conjugates ($k_r$, $M^{-1}$ $min^{-1}$) and reversible inhibition by HI-6 ($K_i$, μM) of human AChE wild type and its Y337A/F338A mutant. All constants (± SD) were determined from at least 3 experiments. Experiments were performed in 0.1 M sodium phosphate buffer containing 0.01 % BSA, pH 7.4, at 25 °C.

| hAChE | $k_a$ | $k_i$ | $K_i$ | $k_r$ |
|---|---|---|---|---|
| wt | 0.37 ± 0.085[a] | 100 ± 0.2 | 20 ± 0.4 | nd[b] |
| Y337A/F338A | 0.014 ± 0.001 | 77 ± 0.01 | 61 ± 12 | 3300 ± 1000 |

[a] at pH 7.0; from Shafferman et al. (1996); constant for Y337A/F338A determined here at pH 7.4, 0.1 M phosphate buffer, at 25 °C

[b] from Cochran et al. (2011); nd - not detected due to a fast aging of soman-hAChE wt conjugate

## Table 2

Antidotal/scavenging efficacy of HI-6 and human AChE mutant Y337A/F338A in soman-exposed mice presented in terms of the protective index (95 % confident limits are given in parentheses) and the maximal dose of poison (MDP).[a,b]

| Pretreatment (*i.v.*) | | Therapy (*i.m.*) | Protective index | MDP[b] |
|---|---|---|---|---|
| 5 min | 15 min | 1 min after soman (*s.c.*) | | |
| | | 110 mg/kg HI-6 with atropine | 4.0 (2.9–5.6) | <3.2 |
| 110 mg/kg HI-6 | | 110 mg/kg HI-6 with atropine | 6.3 (5.3–7.4) | 5.0 |
| 1 mg/kg mutant + 110 mg/kg HI-6 | | 110 mg/kg HI-6 with atropine | 7.1 (5.9–8.5) | 5.0 |
| | 70 mg/kg HI-6 | 110 mg/kg HI-6 with atropine | 6.0 (5.3–6.7) | 5.0 |
| | 1 mg/kg mutant + 70 mg/kg HI-6 | 110 mg/kg HI-6 with atropine | 4.3 (3.3–5.7) | 2.5 |

[a] $LD_{50}$ (*s.c.*) of soman was 136 μg/kg, while $LD_{50}$ of HI-6 was 450 and 283 mg/kg for *i.m.* and *i.v.*, respectively.

[b] MDP (maximal dose of poison) is the maximal dose of the soman $LD_{50}$ that was fully counteracted by the treatment applied.

**Table 3**

Toxicity symptoms in mice pretreated with HI-6 or with HI-6 plus human AChE mutant Y337A/F338A 5 min before soman exposure. Symptoms were observed at selected time points post-soman exposure, intensity of symptoms is graded by number of plus symbols.[a,b]

| Time(min) | 5.0 x $LD_{50}$ soman | | 6.3 x $LD_{50}$ soman | | 7.9 x $LD_{50}$ soman | |
|---|---|---|---|---|---|---|
| | HI-6 | HI-6 + AChE mutant | HI-6 | HI-6 + AChE mutant | HI-6 | HI-6 + AChE mutant |
| 1 | − | − | ++ | − | +++++ | − |
| 5 | ++ | ++ | +++ | ++ | +++++ | + |
| 10 | ++++ | + | ++++ | +++ | +++++ | ++ |
| 30 | ++++ | ++ | ++++ | ++ | +++++ | ++ |
| 60 | +++ | + | ++++ | ++ | +++++ | ++ |
| 90 | ++ | + | ++++ | ++ | +++++ | ++ |
| 120 | + | + | +++++ | ++ | +++++ | ++ |

[a] no symptoms (−), mice were hypoactive (+) with tremor (++), moderate tremor and convulsions (+++), strong tremor and breathing disturbance (++++), or breathing disturbance and no touch-induced reaction (+++++)

[b] doses in pretreatment were 1 mg/kg AChE mutant and 110 mg/kg HI-6. All mice received HI-6 (110 mg/kg) with atropine (10 mg/kg) 1 min after soman exposure. $LD_{50}$ (*s.c.*) of soman was 136 μg/kg.

**Table 4**

Detailed kinetic analysis of reactivation of soman-human AChE mutant Y337A/F338A conjugate by oximes (0.10–3.0 mM). The maximal first-order reactivation rate constant ($k_{+2}$, $min^{-1}$), the dissociation constant of the phosphylated enzyme-oxime reversible complex ($K_{OX}$, μM), and overall second-order reactivation rate constant ($k_r$, $M^{-1}min^{-1}$) were determined by nonlinear regression from reactivation data presented in Fig. 4. All constants (± SD), maximal reactivation ($React_{max}$, %) and the time in that maximal reactivation was reached ($t_{max}$, min), were determined from at least 3 experiments.

| Oxime | $K_i$[a] | $K_{OX}$ | $k_2$ | $k_r$ | $React_{max}$ | $t_{max}$ |
|---|---|---|---|---|---|---|
| HI-6 | 61 ± 12 | 320 ± 100 | 1.0 ± 0.12 | 3300 ± 1000 | 60 | 10 |
| RS-170B | 21 ± 2 | 380 ± 60 | 2.6 ± 0.1 | 7000 ± 870 | 63 | 3 |
| RS-95C | nd[b] | 110 ± 26 | 0.37 ± 0.02 | 3500 ± 770 | 60 | 15 |
| ICD-585 | 42 ± 6 | 100 ± 20 | 0.14 ± 0.01 | 1400 ± 300 | 70 | 20 |
| HS 6 | 220 ± 66 | 320 ± 90 | 0.23 ± 0.02 | 710 ± 200 | 70 | 30 |

[a] dissociation constant of the uninhibited enzyme-oxime reversible complex ($K_i$, μM) determined in the presence of ATCh (0.05–0.5 mM)

[b] nd – not determined