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Amidine−Oximes: Reactivators for Organophosphate Exposure

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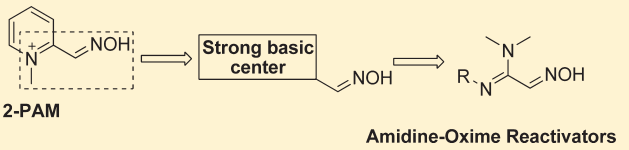
Supporting Information

ABSTRACT: A new class of amidine−oxime reactivators of organophosphate (OP)-inhibited cholinesterases (ChE) were designed, synthesized, and tested. These compounds represent a novel group of oximes with enhanced capabilities of crossing the blood−brain barrier. Lack of brain penetration is a major limitation for currently used oximes as antidotes of OP poisoning. The concept described herein relies on a combination of an amidine residue and oxime functionality whereby the amidine increases the binding affinity to the ChE and the oxime is responsible for reactivation. Amidine−oximes were tested in vitro and reactivation rates for OP-BuChE were greater than pralidoxime (2-PAM) or monoisonitrosoacetone (MINA). Amidine−oxime reactivation rates for OP-AChE were lower compared to 2-PAM but greater compared with MINA. After pretreatment for 30 min with oximes **15c** and **15d** (145 μmol/kg, ip) mice were challenged with a soman model compound. In addition, **15d** was tested in a post-treatment experiment (145 μmol/kg, ip, administration 5 min after sarin model compound exposure). In both cases, amidine−oximes afforded 100% 24 h survival in an animal model of OP exposure.



INTRODUCTION

Organophosphate (OP) nerve agents and pesticides could be used as weapons of mass destruction and represent a potential threat to military personnel and civilians. In addition, farmers and agricultural workers handle large amounts of OPs and are constantly exposed to these toxic materials. Between 150 000 and 300 000 OP-related toxic incidences are reported annually in the United States.[1] OP pesticides (e.g., parathion, chlorpyrifos) and chemical warfare nerve agents (e.g., sarin, soman, tabun, VX) are potent covalent inhibitors of cholinesterase enzymes (ChE) in the blood and in the central nervous system (CNS).[2] The resulting OP−ChE adducts undergo very slow spontaneous hydrolysis and allow acetylcholine (ACh) to build up in the synapses. Accumulation of ACh stimulates autonomic receptors and blocks neuromuscular junction receptors.[3] The symptoms resulting from nerve agent exposure are primarily the consequence of accumulation of excess ACh where ordinarily only small amounts of ACh are present at nerve junctions.[4] Currently, clinically available treatment of acute OP poisoning includes combined administration of a ChE reactivator (i.e., oxime), a muscarinic receptor antagonist (i.e., atropine), and an anticonvulsant (i.e., diazepam). Oximes currently in use for treatment of OP poisoning are functionalized pyridinium salts (Figure 1). Pralidoxime (2-PAM chloride), discovered in 1955,[5] was the first oxime used clinically for treatment of OP poisoning and currently it is a standard antidote in the United States.

Recent efforts in the field of OP reactivation have focused on development of more effective bis-quaternary oximes[6,7] (e.g., HI-6, MMB-4, obidoxime, Figure 1). Elaboration of an oxime as a bis-quaternary compound generally improves its reactivation properties, because the second cation interacts with the peripheral binding site of ChE and increases binding affinity (e.g., for

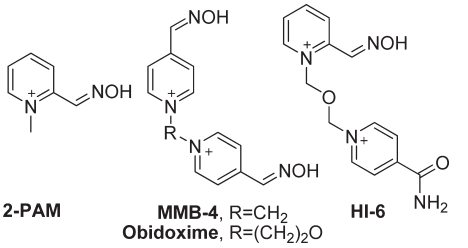


Figure 1. Chemical structures of currently available pyridinium oximes.

AChE, HI-6 K_D = 24 μM, 2-PAM K_D = 210 μM).[8] For example, for sarin-inhibited AChE, HI-6 has a reactivation rate constant (k_r) = 22 000 M^{-1} min^{-1}, whereas the k_r for 2-PAM = 403 M^{-1} min^{-1}.[8] However, introduction of a second positive charge into a pyridinium oxime effectively precludes the compound from entry into the brain via the blood−brain barrier (BBB). Because brain ChEs are the key target for toxic action of OPs, bis-cationic reactivators possess significant deficiencies in reactivating brain ChE's. Treatment of OP poisoning with bis-cationic pyridinium oximes does not provide complete protection from CNS toxicity, and consequently, individuals exposed suffer from chronic neurological side effects. Approximately 20% of the victims of sarin exposure in the Tokyo subway attack of 1995 had symptoms of OP poisoning 1 year after the attack.[9] To address the deficiencies of cationic pyridinium oximes and develop agents that protect the CNS from toxicity of OP exposure, oximes capable of crossing the BBB were developed.

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To date, only a few BBB-penetrable oximes have been reported in the literature. However, none of them have been extensively developed further because of low functional activity and/or significant toxicity.[10,11] Modification of quaternary oximes to improve BBB permeability requires introducing lipophilic substituents to counteract the hydrophilic character of the pyridinium positive charge. The active site serine of ChEs lies at the bottom of a deep, narrow gorge and one possible disadvantage of quaternary oximes with large lipophilic groups might be the lack of space at the active site to accommodate additional bulk. Another disadvantage of this approach is that lipophilic derivatives of 4-PAM have been described to have significant toxicity (i.e., LD_{50} values from 4.2 to 21.9 mg/kg, rats, iv).[11] 2-PAM has been delivered into the CNS for protection by relying on modification of 2-PAM by attaching a glucose molecule. This approach presumably utilizes glucose transporters for BBB delivery.[12] Recent efforts to reactivate CNS ChEs have relied on oxime antidotes with increased lipophilicity and BBB permeability compared to 2-PAM-type reactivators. The uncharged oximes monoisonitrosoacetone (MINA) and diacetylmonooxime (DAM) (Figure 2) are reportedly able to penetrate the BBB but have a much lower propensity for reactivation of OP−ChE in peripheral tissues and blood ChEs compared to 2-PAM and other quaternary oximes.[13,14]

An alternative approach relies on the use of a prodrug of 2-PAM that undergoes oxidation within the CNS to produce a functionally active quaternary oxime (Figure 2).[15−17] However, this approach suffers from two disadvantages: (1) the synthesis of the prodrug is inherently difficult,[18] and (2) pro-2-PAM is unstable to autoxidation. Therefore, novel uncharged reactivators capable of crossing the BBB and reactivating ChEs within the CNS are needed.

Retrostructural analysis of 2-PAM led us to the general structure of novel compounds containing a strongly basic amidine center adjacent to the oxime function (Figure 3).

An amidine group is frequently used in drugs (e.g., dabigatran, diminazen, pentamidine) because of its nontoxic properties, pronounced water solubility, and action as an efficient hydrogen-bond acceptor. In the case of amidine−oxime reactivators (Figure 3), the basic group readily undergoes protonation under physiological conditions to form a positively charged center, similar to a pyridinium quaternary nitrogen. Protonated amidine groups presumably facilitate efficient binding to ChE's through π−cation interactions with aromatic amino acid residues at the ChE active site. The concept of incorporating an amidine group into small molecules to increase a compound's binding affinity to biomolecules was reported in the literature, and amidines have been used as inhibitors of AChE[19,20] and antagonists of the muscarinic acetylcholine receptor.[21]

In the approach developed herein, the amidine moiety supplies a pseudopositive charge responsible for efficient binding to OP-inhibited ChEs, while the proximal oxime group is a nucleophile responsible for reactivation of the phosphylated enzyme. In addition, the electron-withdrawing character of the protonated amidine group enhances ionization of the oxime group and increases the concentration of the functionally active nucleophilic oxime anion (Figure 4).

The pK_a of the oxime group in MINA is 8.3[22] and provides approximately 12% of the oxime in the deprotonated form at pH 7.4, according to the Henderson−Hasselbalch equation. On the basis of the structural similarities between MINA and amidine−oximes and taking into consideration the electron-withdrawing character of the protonated amidine functionality, amidine−oximes herein should be deprotonated at physiological pH equally if not greater than MINA. Therefore, the amidine functionality plays a dual role in amidine−oximes: first as a pseudopositive charge to efficiently bind to an OP-inhibited enzyme and second as a functional group that enhances the nucleophilicity of the oxime.

Herein, we describe a new class of amidine−oxime reactivators of OP−ChEs. Compared to the pro-2-PAM/2-PAM system or MINA, amidine−oximes have advantageous properties including (1) improved chemical stability (compared to pro-2-PAM), (2) greatly improved lipophilicity (compared to quaternary oximes), (3) increased in vitro reactivation efficacy of OP-inhibited BuChE (compared to 2-PAM) and AChE (compared to MINA), and (3) significant protection from the in vivo toxic effects of OPs.

RESULTS AND CHEMISTRY

Because of the paucity of examples in the literature of compounds containing an amidine and oxime functionality in

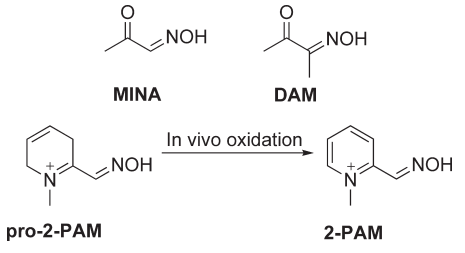

Figure 2. Chemical structures of uncharged oximes MINA and DAM and a prodrug of 2-PAM that undergoes in vivo oxidation to 2-PAM.

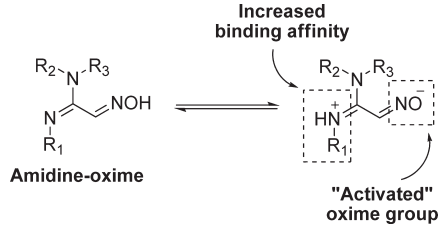

Figure 4. An amidine functional group plays a dual role by providing a positive charge and increasing the ionization of the oxime oxygen atom.

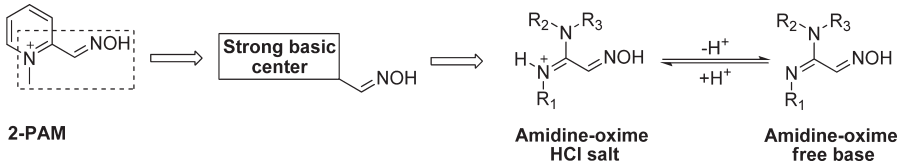

Figure 3. Retrostructural analysis of 2-PAM led to the structure of an amidine−oxime.

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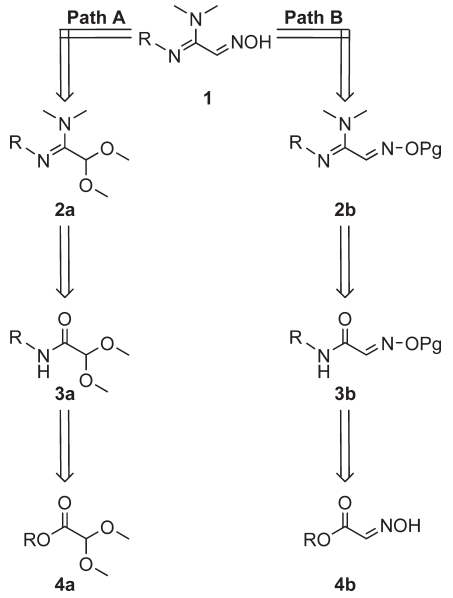
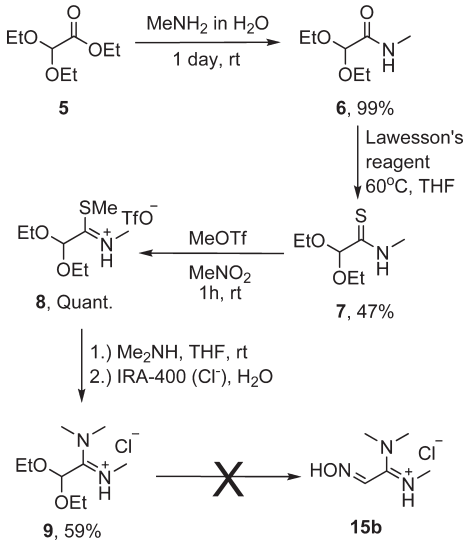


Figure 5. Two retrosynthetic paths for synthesis of amidine−oxime **1**.

Scheme 1. Unsuccessful Synthesis of Amidine−Oxime 15b by Path A



the same molecule, the synthesis of amidine−oximes required a new strategy. Retrosynthetic analysis revealed two possible approaches (Figure 5). In path A, the amidine **2a** was synthesized first and then a protected aldehyde was converted to an oxime **1**. In path B, the starting material was a protected oxime **4b** and then the amidine **2b** was synthesized. Finally, the oxime group was deprotected and the desired product **1** was obtained. Generally, both strategies followed the same synthetic approach but differed in the type of protecting group.

Both approaches (paths A and B, Figure 5) started from readily available esters **4a**,**b** containing either a protected aldehyde or a protected oxime group. The ester function was subsequently modified into amides **3a**,**b** that served as a starting point for making amidine **2a**,**b** using a thioimidate intermediate.[23,24] Application of the Pinner method for synthesis of an amidine group in an amidine−oxime was not considered because it required a hypothetical cyano−oxime that is predicted to be very unstable. The desired product **1** was obtained from **2a** after deprotection and subsequent reaction with hydroxylamine or by deprotection of **2b**. In theory, neither of the two methods had a significant advantage over the other and hence we explored both synthetic routes in parallel.

In path A, ethyl diethoxyacetate **5** was readily converted to amide **6** in the presence of $MeNH_2$ and then to thioamide **7** using Lawesson's reagent (Scheme 1). Activation of the carbonyl group through *S*-methylation of thioamide **7** provided thioimidate **8** in quantitative yield. An attempt to methylate **7** using MeI in acetone required elevated temperature and led to starting material and/or product decomposition. Treatment of **7** with MeOTf in $MeNO_2$ at room temperature gave **8** in quantitative yield. Further treatment with Me_2NH in THF gave amidine **9** with a satisfactory yield (i.e., 59%). The final step was acid-catalyzed deprotection of acetal **9**. No reaction was observed upon treatment of **9** with 4 M HCl at room temperature, and more harsh reaction conditions caused decomposition of the starting material and/or product.

In path B, benzyl-protected ethyl glyoxylate oxime **10** was synthesized as described before.[25] Ester **10** was treated with ammonia or alkyl amine $MeNH_2$, $EtNH_2$, $PrNH_2$ to afford **11a**−**d** (Scheme 2). Thioamides **12a**−**d** were obtained by treating **11a**−**d** with Lawesson's reagent in good yield. The triflate salts of **13a**−**d** were prepared by *S*-methylation of **12a**−**d** using MeOTf. The desired amidines **14a**−**d** were obtained by treating **13a**−**d** with Me_2NH in THF according to a previously established procedure shown in Scheme 1.

An initial attempt to remove the benzyl group of **14a**−**d** using $Pd/C/H_2$ was unsuccessful and only starting material was recovered, even after using an equivalent amount of the catalyst, probably because of sulfur-based impurities from the previous step that poisoned the catalyst. Extensive chromatographic purification of **14a**−**d** did not solve this problem. To overcome this issue, sulfur impurities were removed using in situ prepared fine powder of silver metal. Amidine hydrochlorides **14a**−**d** were dissolved in H_2O and then added to a solution containing an equivalent amount of $AgNO_3$. The precipitated AgCl quickly underwent decomposition upon exposure to light and formed a black silver powder. Metallic silver helped to remove sulfur impurities from the previous synthetic step and prevented poisoning of the Pd catalyst in the debenzylation step. Debenzylation of highly purified amidine chlorides **14b**−**d** was efficient, and in the case of methyl-substituted amidine **14b**, the desired amidine−oxime **15b** was obtained in 97% yield. In the case of ethyl and propyl amidine−oximes **14c**,**d**, debenzylation provided products **15c**,**d** with much lower yields, probably because of reduction of the oxime group. Deprotection of unsubstituted amidine−oxime **14a** using the method described in Scheme 2 was unsuccessful, and only reduction of the oxime group was observed. Debenzylation of **14a** was not successful using different catalysts (i.e., Pd/C, $Pd/BaSO_4$, $Pd/CaCO_3$/5%Pb) and solvents (EtOH, THF, EtOAc). However, it was observed that strong Lewis acids such as anhydrous $AlCl_3$ in CH_2Cl_2 removed the benzyl group of **14a** to afford amidine−oxime **15a** in good yield (Scheme 2).

Amidine−oxime hydrochlorides **15a**−**d** were tested for chemical stability and showed no sign of decomposition after 3 months at −18 °C, as judged by NMR. However, the free base form of **15a**−**d** underwent decomposition in CD_2Cl_2, and after 5

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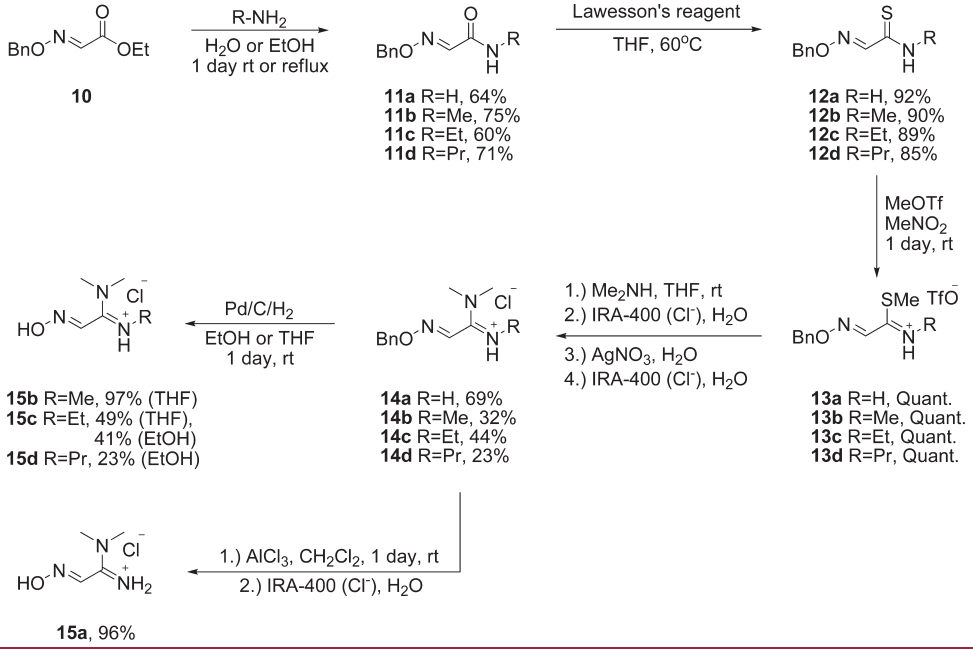
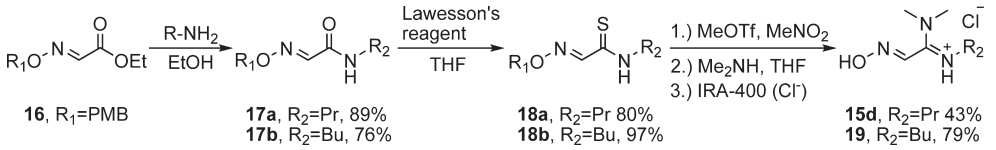
Scheme 2. Synthesis of *N*,*N*′,*N*″-Trisubstituted Amidine−Oximes 15a−d



Scheme 3. Synthesis of Amidine−Oximes 15d and 19 Using a PMB Protecting Group



days, significant amounts of impurities were detected by NMR. The synthetic method was successfully scaled-up to prepare over 200 mg of amidines **15a−d**. However, debenzylation of **14b−d** was challenging, and extensive purification from sulfur impurities was always required to obtain satisfactory yields of **15b−d**. Difficulties in removing the benzyl group encouraged the search for an alternative protecting group that could be removed under mild conditions besides hydrogenation. From a broad panel of protecting groups, *p*-methoxybenzyl (PMB) was selected, because it could be removed using significantly milder conditions. To test the utility of the PMB group in the synthesis, propyl amidine−oxime **15d** was chosen, as it gave a low yield in the debenzylation step. PMB-protected ethyl glyoxylate oxime **16** was converted as above to the amide **17** and thioamide **18** derivatives (Scheme 3).

Unexpected results were obtained during the *S*-methylation of **18a**, where traces of water likely caused hydrolysis of MeOTf, forming triflic acid in situ. Under acidic conditions the removal of the PMB group occurred, and therefore, the crude reaction mixture was directly used in the amidine synthesis. Using this two-step procedure (Scheme 3), the final product **15d** was obtained in 31% overall yield compared with 3.2% following the method of Scheme 2. A similar methodology shown in Scheme 3 was used to prepare the *N*-butyl-substituted amidine−oxime **19**, and in this case the product was isolated in greater than 58% overall yield.

Determination of the pK_a for 15c. To support and verify the hypothesis that a protonated amidine group decreases the pK_a of the oxime function and also to establish the amount of amidine−oxime that exists in the zwitterionic form at physiological pH, the pK_a values for one representative compound, **15c**, were determined. A series of 50 mM buffer solutions with pH from 3 to 13.5 and constant ionic strength (300 mM) was prepared. A solution of **15c** was added to each buffer and the UV spectrum was recorded in the range of 220−700 nm. The change in absorbance at 260 nm was plotted versus pH to obtain the pK_a profile (Figure 6).

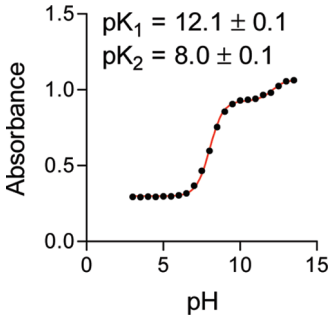


Figure 6. pK_a determination for **15c**.

On the basis of the results for **15c**, the pK_a for the oxime group is 8.0 ± 0.1, whereas the pK_a for the amidine group is 12.1 ± 0.1. Therefore, at physiological conditions, pH 7.4, the amidine group is almost exclusively protonated, whereas the oxime group is deprotonated to approximately 25 ± 5%, based on the

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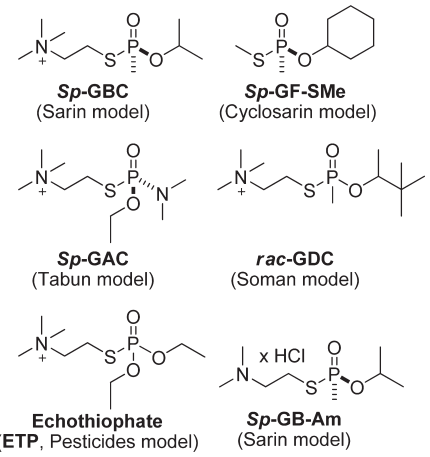

Figure 7. Chemical structures of nerve agent model compounds used in these studies.[28,29]

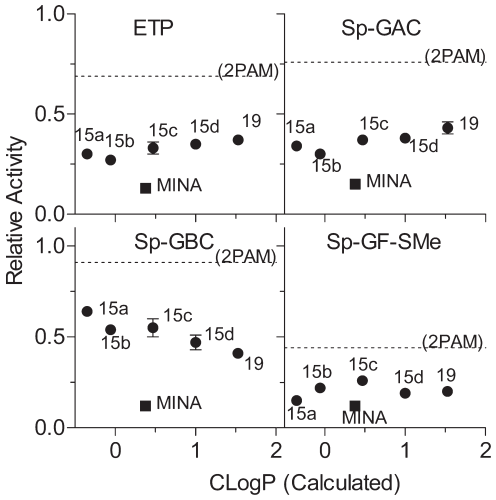

Figure 8. Reactivation of OP-inhibited AChE by amidine−oximes **15** and **19**, MINA, and 2-PAM. Enzyme was inhibited with OP prior to incubation with 100 μM oxime, as described in the Experimental Section. After 60 min, the recovered AChE activity was determined by measuring AChE esterase functional activity. Data depicted shows the average and standard deviation (as a fraction of noninhibited control samples) for AChE activity (n = 2 incubations per oxime). Unless otherwise indicated, the standard deviation bars were smaller than the data markers. The broken (dashed) lines indicate the relative activity of samples incubated with 2-PAM at 100 μM. Relative activity values of enzyme-inhibited samples incubated in the absence of oxime were ≤0.03.

Henderson−Hasselbalch equation. In comparison, MINA has a pK_a = 8.3[22] and approximately 12% of the oxime exists in the deprotonated form. On the basis of the literature, an optimal pK_a for the oxime group should be approximately 7.8,[26] or within the range 7.5−8.0.[27] Oxime reactivators with pK_a values lower than 7.5 are too weakly nucleophilic, whereas a pK_a above 8.0 does not provide enough of the oxime anion form at physiological conditions. Amidine−oxime **15c** fulfills the requirement of an optimal pK_a for efficient reactivation, and its value is above that of currently available quaternary oximes, 2-PAM (pK_a = 7.68), HI-6 (pK_a = 7.28), obidoxime (pK_a = 7.78), and MMB-4 (pK_a = 7.3).[27] The difference between pK_a values for 2-PAM and **15c** comes from the greater electron-withdrawing character of the pyridinium quaternary group compared to the protonated amidine in **15c**. Although the pK_a of the oxime group in **15c** is less favorable than the pK_a of a quaternary oxime, the lack of a permanent positive charge makes amidine−oximes orders of magnitude more lipophilic, and therefore, it has a greater propensity to enter the CNS.

In Vitro Biochemical Studies. The ability of oximes **15a−d** and **19** to reactivate human AChE and BuChE inhibited by nerve agent model compounds (i.e., model compounds of sarin, cyclosarin, tabun)[28,29] and pesticides (i.e., echothiophate) (Figure 7) was evaluated by incubating OP-inhibited enzyme with oxime for 20−60 min and then comparing the enzyme's esterase activity to a noninhibited control sample.

During the course of the experiment, enzyme sample was incubated with oxime, and then an aliquot was diluted 20-fold into a solution containing substrate to assess the amount of esterase functional activity. The dilution carried with it free oxime, yielding a concentration of 5 μM in the esterase functional activity assay. To ensure that this concentration of oxime was neither inhibiting enzyme nor nonenzymatically increasing the apparent esterase activity, oxime concentrations were titrated over a range of concentrations (i.e., 3−100 μM) in enzyme incubations with 1 mM substrate and the observed rates of turnover were recorded. For all but the most lipophilic compound (i.e., **19**), the catalytic activity observed in the presence of 100 μM amidine−oxime was still within 20% of the no amidine−oxime control sample, and no further analysis was conducted. Amidine−oxime **19** inhibited AChE with an IC_{50} value of 54 ± 8 μM (see Supporting Information). On the basis of this value, it is estimated that 5 μM amidine−oxime present in the kinetic assay following AChE reactivation trials would yield less than 10% inhibition, and this putative inhibitory effect was therefore disregarded when analyzing reactivation levels. Lack of inhibition was seen for BuChE with oximes **15a−d**. Compared to studies with AChE, oxime **19** bound less tightly to BuChE, with an IC_{50} value estimate larger than 300 μM.

Reactivation studies of amidine−oximes **15a−d** and **19** were evaluated together with two reference compounds (i.e., 2-PAM and MINA). Compared with uncharged reactivator MINA, amidine−oximes **15a−d** and **19** were more potent reactivators. A plot of reactivated ChEs activity (relative to a noninhibited enzyme sample) versus calculated ClogP showed no significant dependence on reactivator structure in the presence of ***Sp*-GAC** or ***Sp*-GF-SMe** (Figure 8). In the presence of **ETP**, a slight but discernible increase in AChE reactivation was observed in going from hydrogen- (**15a**) to butyl-substituted (**19**) amidine−oximes. In contrast, an inverse relationship was observed for AChE inactivated with ***Sp*-GBC**. Initial studies with AChE showed less than 10% functional activity was recovered after 20 min incubation time with amidine−oximes **15a−d** and **19** (see Supporting Information), and therefore, the incubation time was increased to 60 min. The increased duration of incubation improved the levels of reactivation, and most amidine−oximes yielded less than 60% reactivation (Figure 8). The lowest degree of reactivation was observed for GF-inhibited enzyme. In the presence of AChE, the quaternary reactivator 2-PAM was a more potent reactivator than MINA or amidine−oximes **15a−d** and **19** (Figure 7).

In contrast to AChE, reactivation of BuChE showed a considerably larger dependence on amidine−oxime structure (Figure 9). A plot of relative percent remaining functional activity

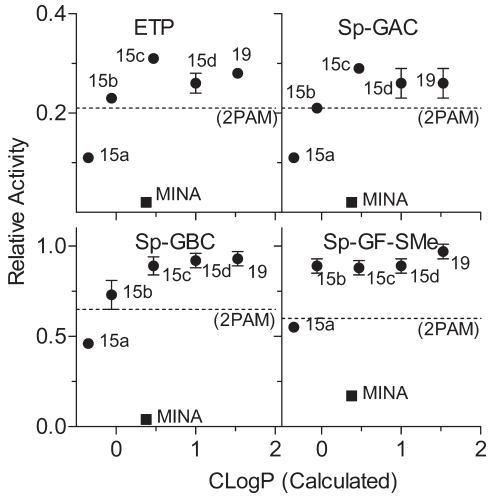

Figure 9. Reactivation of OP-inhibited BuChE by amidine−oximes **15** and **19**, MINA, and 2-PAM. Enzyme was inhibited with OP prior to incubation with 100 μM amidine−oxime, as described in the Experimental Section. After 20 min, the recovered BuChE functional activity was determined by measuring BuChE activity. Data depicted the average and standard deviation (as a fraction of noninhibited control samples) for BuChE activity (n = 2 incubations per oxime). Unless otherwise indicated, the standard deviation bars were smaller than the data markers. The broken (dashed) lines indicate the relative activity of samples incubated with 2-PAM at 100 μM. Relative activity values of enzyme-inhibited samples incubated in the absence of oxime were ≤0.03 for **ETP**, ***Sp*-GAC**, and ***Sp*-GBC**. The corresponding ***Sp*-GFC** sample had a value of 0.06.

of BuChE versus ClogP showed a modest increase in ***Sp*-GAC** reactivation in going from a hydrogen- (**15a**) to an *N*-butyl-substituted (**19**) amidine−oxime but a strong dependence on increasing lipophilicity in the presence of ***Sp*-GBC** or ***Sp*-GF-SMe**. In the presence of **ETP**, a parabolic relationship was observed between lipophilicity of amidine−oximes **15** and **19** and BuChE reactivation. ***Sp*-GBC**- and ***Sp*-GF-SMe**-inhibited BuChE recovered over 60% functional activity within a 20 min reactivation period for the most lipophilic *N*-butyl amidine−oxime **19**. Amidine−oximes **15c**, **15d**, and **19** all showed comparable or greater levels of BuChE reactivation compared to 2-PAM. These results illustrated that amidine−oximes with more lipophilic substituents on the amidine group markedly increased reactivation of ***Sp*-GBC**- and ***Sp*-GF-SMe**-inhibited BuChE. For **ETP** and ***Sp*-GAC**, 2-PAM showed greater reactivation than **15a**−**d** and **19**.

The amidine−oximes showed reactivation rates for OP-AChE within 2−2.5-fold of 2-PAM at equal oxime concentrations (i.e., 100 μM). However, the amidine−oximes are orders of magnitude more lipophilic than 2-PAM and are predicted to enter the brain in a correspondingly improved fashion. The hypothesis is that improved apparent BBB penetration and greater brain concentration improved the concentration of amidine−oximes necessary for reactivation compared to 2-PAM, despite lower rates of reactivation and afford protection from OPs in vivo (see below).

In Vivo Studies. To test the protection of amidine−oximes to exposure to OPs in in vivo experiments, nerve agent model compounds of soman and sarin (i.e., ***rac*-GDC** and ***Sp*-GB-Am**, Figure 7) were used. A nerve agent model of soman was selected because it is known in the literature to be a particularly challenging

Table 1. Effect of Vehicle, 2-PAM, or Amidine−Oximes 15c and 15d on the Lethality of *rac*-GDC

			***rac*-GDC**		
experiment	treatment[a]	μmol	mg/kg	μmol	survival (24 h)
1	vehicle	–	0.25	0.61	2/14
	15c (100 mg/kg)[b]	557			6/6
2	2-PAM (25 mg/kg)	145	0.25	0.61	6/8
	15c (26 mg/kg)[b]				6/6
	15d (28 mg/kg)[b]				6/6
3	2-PAM (6.25 mg/kg)	36	0.25	0.61	0/6
	15c (6.5 mg/kg)[b]				4/6

[a] Mice were pretreated (ip) with vehicle or an amidine−oxime 30 min prior to ***rac*-GDC**. [b] Oximes **15c** and **15d** were administered as hydrochloride salts in isotonic saline. P values for expt 1 = 0.0007, expt 2 = 0.47, expt 3 = 0.06.

organophosphate to reactivate.[30,31] Soman-inactivated ChEs rapidly undergo a P−O-dealkylation processes (i.e., aging) within minutes.[32] On a molecular level, this leaves a negatively charged P−O^- moiety that is resistant to nucleophilic attack and reactivation by oximes. None of the currently available oxime reactivators are able to reactivate an aged ChE, and therefore, reliable in vitro reactivation studies of soman−ChEs cannot be conducted or require elaborated methodology.[33] A nerve agent model of sarin (i.e., ***Sp*-GBC**) was utilized because in vitro studies reported above showed efficient reactivation of AChE (approximately 50%, Figure 8) and BuChE (approximately 80%, Figure 9).

For the in vivo studies using ***rac*-GDC**, compounds **15c**,**d** were selected and three sets of experiments were conducted. Mice were pretreated (ip injection) with vehicle or amidine−oxime 30 min prior to soman model compound (***rac*-GDC**, Figure 7) administration. In human clinical use, im administration is the preferred route of administration; however, this method is not recommended for mouse studies, because of the paucity of muscle mass in a mouse. The 30 min pretreatment regimen (i.e., the time between amidine−oxime pretreatment and OP administration) was chosen because this was estimated to be the time point of peak plasma concentrataion of the amidine−oxime. This estimate was based in part on literature information for the bis-quaternary oxime K-48 (CLogP = −8.2).[34] Peak plasma concentration of K-48 was achieved after approximately 15 min. In the animal paradigm used herein, after OP administration, mice were observed for neurological toxicity symptoms (i.e., seizures, convulsions, muscles twitching, salivation, and rhinorrhea). After 24 h the survival was recorded, and animals were sacrificed by cervical dislocation. In a preliminary experiment, animals were either pretreated with vehicle or **15c** at a dose of 100 mg/kg (ip) to simultaneously evaluate protection from OP and possible acute toxicity of **15c**. No apparent toxicity was observed for **15c** administered alone at a dose of 100 mg/kg (ip), and all animals pretreated with **15c** (100 mg/kg, ip, 30 min prior administration) survived 24 h after OP exposure (Table 1). We estimate that the LD_{50} of amidine−oximes far exceeds 100 mg/kg, but this point was not thoroughly examined. For vehicle-treated animals, only 2/14 control animals survived to the 24 h time point. In a second experiment, animals were pretreated (ip) with either amidine−oximes **15c**,**d** or 2-PAM at the same molar concentration (i.e., 145 μmol; 26 mg/kg for **15c**, 28 mg/kg for **15d**, and 25 mg/kg for 2-PAM). In the second experiment,

Table 2. Effect of Vehicle, MINA, or Amidine−Oxime 15d on the Lethality of *Sp*-GB-Am

			Sp-GB-Am		
experiment	treatment[a]	μmol	mg/kg	μmol	survival (24 h)
1	vehicle	−	0.08	0.305	2/6
	MINA (12.6 mg/kg)	145			3/6
	15d (28 mg/kg)[b]				6/6

[a] Mice were treated (ip) with ***Sp*-GB-Am** and 5 min later vehicle, amidine−oxime **15d**, or MINA was administered. [b] Oxime **15d** was administered as a hydrochloride salt in isotonic saline. *P* values for expt 1 (vehicle vs **15d**) = 0.06, (MINA vs **15d**) = 0.18.

amidine−oximes **15c,d** provided complete protection to animals, compared to 2-PAM, which protected 6/8 mice. In the third experiment, animals were pretreated (ip) with only 25% of the previous oxime dose (i.e., 6.5 mg/kg for **15c** and 6.25 mg/kg for 2-PAM). In this experiment, none of the animals from the 2-PAM-treated group survived, whereas **15c** protected 4/6 animals (Table 1).

We sought to extend the protection studies to another nerve agent model compound to examine the universality of the protective effects of amidine−oximes. In all likelihood, in the case of a real life situation, the OP-exposed victim will be treated with oxime antidotes after OP exposure (post-treatment). To verify whether amidine−oximes are able to protect animals from this more challenging post-treatment situation, mice were exposed to sarin model compound (***Sp*-GB-Am**, Figure 7) and 5 min later the vehicle, MINA (12.6 mg/kg, 145 μmol/kg, i.p.), or **15d** (28 mg/kg, 145 μmol/kg, ip) was administered. Animals were observed for neurological toxicity symptoms as above, and after 24 h, survival was recorded (Table 2). MINA was used as a control because this is currently the only known BBB-penetrating oxime commercially available to compare efficacy with amidine−oxime **15d**.

As shown in Table 2, for vehicle-treated animals 2/6 survived 24 h and significant CNS toxicity was observed (i.e., strong seizures, lack of spontaneous activity, and no interest in food consumption). Similar symptoms were observed for the MINA-treated group, which protected 3/6 animals. After administration of amidine−oxime **15d**, all OP-treated mice survived the treatment and showed behavioral activity comparable with healthy, untreated animals.

DISCUSSION AND CONCLUSIONS

In summary, novel amidine−oximes were designed and prepared using thioimidate intermediates using two different protected oxime functionalities. Use of *p*-methoxybenzyl (PMB) instead of benzyl (Bn) as a protecting group significantly improved the synthetic efficiency of amidine−oximes. Oximes **15a−d** and **19** were tested in vitro for reactivation of inhibited AChE and BuChE in the presence of nerve agent model compounds and pesticide ETP. On the basis of these results, the amidine−oximes examined had superior functional activity compared with MINA for reactivation of inhibited AChE and BuChE. Increase in the lipophilic character of the substituent on the amidine group of the amidine−oximes from hydrogen atom (for **15a**) to *N*-butyl (for **19**) consistently increased the reactivation potency for ***Sp*-GBC**- or ***Sp*-GF-SMe**-inhibited BuChE. The most lipophilic compounds, **15c,d** and **19** (i.e., an increase in CLogP from +0.5 to +1.5), had reactivation potency of ***Sp*-GBC**- or ***Sp*-GF-SMe**-inhibited BuChE comparable to or greater than that of 2-PAM (CLogP −3.7). Despite the fact that the amidine−oximes examined did not afford larger reactivation rates than 2-PAM for OP-inhibited AChE, two amidine−oximes were efficacious in vivo. For example, pretreatment with amidine−oximes **15c,d** (i.e., 30 min prior to OP administration, ip) was able to protect mice from ***rac*-GDC** toxicity. In a post-treatment experiment, animals were exposed to the sarin nerve agent model compound ***Sp*-GB-Am** and treated 5 min later with **15d** or MINA. The group of mice treated with the amidine−oxime survived 24 h and had only minor signs of CNS toxicity, whereas in the case of MINA or vehicle only 3/6 or 2/6 animals, respectively, survived. In the latter case, very severe neurotoxicity symptoms were observed for the vehicle- or MINA-treated animals. We hypothesize that even though amidine−oximes are less potent than 2-PAM in reactivation of OP-AChEs in vitro, they are orders of magnitude more lipophilic than 2-PAM and achieve much greater concentrations in the brain and effectively protect against either pre- or post-treatment of toxic OPs. Additionally, because both enzymes AChE and BuChE are present in the brain,[35] efficient reactivation of BuChE by amidine−oximes **15c** and **15d** likely also contributed to the protection of OP-treated animals. We conclude that treatment of mice with amidine−oximes **15c,d** delivered the compound to the plasma and to the CNS and protected animals from CNS toxicity.

The concept of using amidine−oximes as an alternative to quaternary oximes (e.g., 2-PAM) to more efficiently protect the brain from toxicity of OPs needs to be examined in additional animal models.

EXPERIMENTAL SECTION

General. All reagents and solvents were used as received from commercial sources. Buffers were purchased from VWR Scientific, Inc. (San Diego, CA) in the highest purity commercially available. Synthetic products were isolated using a flash column chromatography system (Teledyne ISCO, CombiFlash Rf) with UV detection at 254 nm. NMR spectra were recorded at 300 MHz (^{1}H) on a Varian Mercury 300 or at 125 MHz (^{13}C) on a Bruker AMX-500 II (NuMega Resonance Lab, San Diego, CA) in $CDCl_3$ or DMSO-d_6, respectively. Chemical shifts were reported as ppm (δ) relative to $CDCl_3$ at 7.26 ppm and DMSO-d_6 at 2.50 or 39.52 ppm. Low-resolution mass spectra were obtained using an Hitachi M-8000 mass spectrometer with an ESI source. Elemental analyses were obtained using a Perkin-Elmer PE2400-Series II, CHNS/O analyzer (NuMega Resonance Lab, San Diego, CA). Calculated ClogP values were determined using ChemDraw Ultra 11.0. Melting points were determined using Mettler Toledo FP62 and were uncorrected. UV−vis spectra were recorded using a Nanodrop 1000 spectrophotometer from Thermo Scientific. The pK_a values were calculated from the correlation of absorbance/pH using GraphPad Prism 5.0.

Purity Determination. Purity of products was determined by a Hitachi 8000 LC−MS (Hitachi, San Jose, CA) using reverse phase chromatography (C18 column, 50 × 4.6 mm, 5 μm, Thomson Instrument Co., Oceanside, CA). Compounds were eluted using a gradient elution of 95/5 to 5/95A/B over 10 min at a flow rate of 1.5 mL/min, where solvent A was aqueous 0.05% TFA and solvent B was acetonitrile (0.05% TFA). For purity data, peak area percent for the TIC, at 210 or 254 nm, and retention time (t_R in minutes) are provided (see Supporting Information). Purity of products was ≥95%. Purity for certain compounds was determined using combustion analysis. Thioimidates **8** and **13a−d** decompose in protic solvents and were used in the synthesis without further purification and purity determination.

Caution! Nerve agent model compounds used in these studies are toxic and must be handled with extreme care by well-trained personnel. Use of these materials has been approved by NIH and DOD. After reactivation studies, biochemical samples were neutralized by stirring with 2 M NaOH/EtOH for 12 h. Remaining solutions were brought back to pH ~7 and disposed of in chemical waste.

2,2-Diethoxy-*N*-methylacetamide (6). A mixture of ethyl diethoxyacetate (15 g, 85.2 mmol) and $MeNH_2$ (40% in H_2O, 15 mL) was stirred at room temperature (rt) overnight. The mixture was evaporated and a clear oil was used without further purification (13.6 g, 99%). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 6.62 (br s, 1H), 4.79 (s, 1H), 3.72−3.53 (m, 4H), 2.84 (d, J = 4.8 Hz, 3H), 1.24 (t, J = 7.1 Hz, 6H).

2,2-Diethoxy-*N*-methylethanethioamide (7). A mixture of **6** (12 g, 74.5 mmol) and Lawesson's reagent (18.1 g, 44.7 mmol, 0.6 equiv) in anhydrous THF (150 mL) was stirred at 60 °C 1 h and then evaporated. The residue was extracted using hexanes:EtOAc (9:1, 5 × 100 mL). Combined organic layers were evaporated, and the residue was purified by column chromatography (silica gel, hexanes→9:1, hexanes:EtOAc) to give a yellow oil (6.2 g, 47%). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.41 (br s, 1H), 5.08 (s, 1H), 3.72−3.58 (m, 4H), 3.19 (d, J = 4.8 Hz, 3H), 1.25 (t, J = 7.1 Hz, 6H).

Methyl 2,2-Diethoxy-*N*-methylethanimidothioate Trifluoromethanesulfonate Salt (8). To a solution of **7** (3 g, 17 mmol) in $MeNO_2$ (50 mL) at rt was added MeOTf (1.9 mL, 2.8 g, 17 mmol). The mixture was stirred at rt for 1 h and then evaporated to give a thick yellow oil (5.8 g, 100%). The crude product was used in the next step without further purification. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 12.08 (br s, 1H), 5.64 (s, 1H), 3.83−3.71 (m, 4H), 3.33 (d, J = 5.1 Hz, 3H), 2.98 (s, 3H), 1.29 (t, J = 7.2 Hz, 6H).

2,2-Diethoxy-*N*,*N*,*N*′-trimethylacetimidamide Hydrochloride Salt (9). To a solution of **8** (3.6 g, 10.5 mmol) in anhydrous THF (10.5 mL) was added Me_2NH (2 M in THF, 10.5 mL, 21 mmol, 2 equiv). The mixture was stirred at rt overnight and then evaporated. The crude oil was purified by column chromatography (silica gel, CH_2Cl_2→9:1, CH_2Cl_2:MeOH) to give a brown oil as the triflate salt. The product was dissolved in a small amount of H_2O and passed through an ion-exchange resin column IRA-400 (Cl^-). The aqueous layer was washed with CH_2Cl_2 (2 × 50 mL) and evaporated to give a yellow oil (1.4 g, 59%). ^{1}H NMR (300 MHz, DMSO-d_6) δ = 9.02 (br s, 1H), 5.71 (s, 1H), 3.84−3.63 (m, 4H), 3.25−3.07 (m, 9H), 1.18 (t, J = 7.1 Hz, 6H). ESI MS (MeOH) $[M + H]^+$ = 188.67 Da.

2-(Benzyloxyimino)acetamide (11a). A mixture of **10**[25] (2 g, 9.6 mmol) and 25% $NH_3 \times H_2O$ (10 mL) was stirred at rt overnight. The reaction was filtered and the product was obtained as a white solid (1.1 g, 64%). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.48−7.27 (m, 6H), 6.39 (br s, 1H), 5.59 (br s, 1H), 5.20 (s, 2H).

2-(Benzyloxyimino)-*N*-methylacetamide (11b). A mixture of **10** (10 g, 48.3 mmol) and $MeNH_2$ (40% in H_2O, 10 mL) was stirred at rt overnight and then diluted with H_2O (50 mL) and filtered. The product was washed with a small amount of Et_2O to give a yellow solid (7 g, 75%). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.48 (s, 1H), 7.43−7.29 (m, 5H), 6.46 (br s, 1H), 5.18 (s, 2H), 2.88 (d, J = 6.8 Hz, 3H).

2-(Benzyloxyimino)-*N*-ethylacetamide (11c). A mixture of **10** (5 g, 24 mmol) and $EtNH_2$ (2 M in MeOH, 24 mL, 48 mmol, 2 equiv) was stirred at 60 °C overnight and then evaporated. The residue was purified by column chromatography (silica gel, hexanes→7:3, hexanes:EtOAc) to give a yellow solid (3 g, 60%). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.47 (s, 1H), 7.40−7.29 (m, 5H), 6.42 (br s, 1H), 5.19 (s, 2H), 3.40−3.32 (m, 2H), 1.19 (t, J = 7.2 Hz, 3H).

2-(Benzyloxyimino)-*N*-propylacetamide (11d). A mixture of **10** (5 g, 24.1 mmol) and $PrNH_2$ (3.9 mL, 2.8 g, 48.2 mmol, 2 equiv) in anhydrous EtOH (50 mL) was stirred at 60 °C 1 day and then evaporated. The residue was purified by column chromatography (silica gel, hexanes→7:3, hexanes:EtOAc) to give a yellow solid (3.8 g, 71%). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.47 (s, 1H), 7.45−7.28 (m, 5H), 6.48 (br s, 1H), 5.19 (s, 2H), 3.28 (q, J = 6.9 Hz, 2H), 1.62−1.51 (m, 2H), 0.94 (t, J = 7.2 Hz, 3H).

2-(Benzyloxyimino)ethanethioamide (12a). A mixture of **11a** (3 g, 16.8 mmol) and Lawesson's reagent (4.1 g, 10.1 mmol, 0.6 equiv) in anhydrous THF (150 mL) was stirred at 60 °C 1 h and then evaporated. The crude material was purified by column chromatography (silica gel, hexanes→9:1, hexanes:EtOAc) to give a yellow solid (3 g, 92%). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.78 (s, 1H), 7.42−7.31 (m, 5H), 5.21 (s, 2H).

2-(Benzyloxyimino)-*N*-methylethanethioamide (12b). The title compound was obtained similarly to **12a** (yellow solid, 90%). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.25 (br s, 1H), 7.84 (s, 1H), 7.41−7.31 (m, 5H), 5.19 (s, 2H), 3.22 (d, J = 5.1 Hz, 3H).

2-(Benzyloxyimino)-*N*-ethylethanethioamide (12c). The title compound was obtained similarly to **12a** (yellow solid, 89%). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.18 (br s, 1H), 7.81 (s, 1H), 7.42−7.28 (m, 5H), 5.20 (s, 2H), 3.78−3.67 (m, 2H), 1.31 (t, J = 7.2 Hz, 3H).

2-(Benzyloxyimino)-*N*-propylethanethioamide (12d). The title compound was obtained similarly to **12a** (yellow solid, 85%). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.19 (br s, 1H), 7.81 (s, 1H), 7.41−7.27 (m, 5H), 5.19 (s, 2H), 3.69−3.62 (m, 2H), 1.78−1.66 (m, 2H), 0.99 (t, J = 7.5 Hz, 3H).

Methyl 2-(Benzyloxyimino)ethanimidothioate Trifluoromethanesulfonate Salt (13a). To a solution of **12a** (2.6 g, 13.4 mmol) in $MeNO_2$ (30 mL) at rt was added MeOTf (1.6 mL, 2.4 g, 14.7 mmol, 1.1 equiv). The mixture was stirred at rt overnight and then evaporated to give a dark red solid (4.8 g, quant.). Crude product was used in the next step without further purification. ^{1}H NMR (300 MHz, $CDCl_3$) δ = 11.24 (br s, 1H), 10.70 (br s, 1H), 8.25 (s, 1H), 7.41−7.32 (m, 5H), 5.35 (s, 2H), 2.71 (s, 3H).

Methyl 2-(Benzyloxyimino)-*N*-methylethanimidothioate Trifluoromethanesulfonate Salt (13b). The title compound was obtained similarly to **13a** (yellow solid, quant.). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 12.23 (br s, 1H), 8.29 (s, 1H), 7.40−7.34 (m, 5H), 5.37 (s, 2H), 3.35 (d, J = 5.1 Hz, 3H), 2.72 (s, 3H).

Methyl 2-(Benzyloxyimino)-*N*-ethylethanimidothioate Trifluoromethanesulfonate Salt (13c). The title compound was obtained similarly to **13a** (red oil, quant.). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 12.26 (br s, 1H), 8.36 (s, 1H), 7.40−7.29 (m, 5H), 5.36 (s, 2H), 3.76−3.66 (m, 2H), 2.67 (s, 3H), 1.50−1.43 (m, 3H).

Methyl 2-(Benzyloxyimino)-*N*-propylethanimidothioate Trifluoromethanesulfonate Salt (13d). The title compound was obtained similarly to **13a** (yellow oil, quant.). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 12.22 (br s, 1H), 8.37 (s, 1H), 7.41−7.30 (m, 5H), 5.36 (s, 2H), 3.62 (q, J = 6.6 Hz, 2H), 2.66 (s, 3H), 1.96−1.82 (m, 2H), 1.02 (t, J = 7.6 Hz, 3H).

2-(Benzyloxyimino)-*N*,*N*-dimethylacetimidamide Hydrochloride Salt (14a). To a solution of **13a** (2.1 g, 5.8 mmol) in anhydrous THF (25 mL) was added Me_2NH (2 M in THF, 5.8 mL, 11.6 mmol, 2 equiv). The mixture was stirred at rt for 10 min and then evaporated. The crude oil was purified by column chromatography (silica gel, CH_2Cl_2→9:1, CH_2Cl_2:MeOH) to give a brown oil of the triflate salt. The product was dissolved in small amount of hot 5% MeOH in H_2O and passed through an ion-exchange resin column IRA-400 (Cl). The aqueous layer was washed with Et_2O (1 × 100 mL) and evaporated to give a brown oil. The product was then stirred with anhydrous THF and changed into a white solid (980 mg, 69%). ^{1}H NMR (300 MHz, DMSO-d_6) δ = 9.18 (br s, 1H), 8.85 (br s, 1H), 8.39 (s, 1H), 7.51−7.30 (m, 5H), 5.30 (s, 2H), 3.21 (s, 3H), 3.13 (s, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ = 155.9, 140.1, 136.1, 128.6, 128.5, 128.4, 77.4, 40.6, 33.9. ESI MS for $[M + H]^+$ = 205.95 Da.

2-(Benzyloxyimino)-*N*,*N*,*N*′-trimethylacetimidamide Hydrochloride Salt (14b). To a solution of **13b** (5.7 g, 15.4 mmol)

in anhydrous THF (15 mL) was added Me_2NH (2 M in THF, 15.4 mL, 30.8 mmol, 2 equiv). The mixture was stirred at rt overnight and then evaporated. The crude oil was suspended in a small amount of hot H_2O and passed through an ion-exchange resin column IRA-400 (Cl). The aqueous layer was washed with CH_2Cl_2 (2 × 50 mL) and evaporated. The crude **14b** (2.6 g, 10.2 mmol) was dissolved in H_2O (10 mL) and a solution of $AgNO_3$ (1.7 g, 10.2 mmol) in H_2O (10 mL) was added. The mixture was stirred at rt exposed to sunlight for 1 h and then filtered and evaporated. The crude oil was purified by column chromatography (silica gel, 9:1, CH_2Cl_2:MeOH) to give a white solid of **14b** as the nitrate salt (1.4 g, 32%). The **14b** chloride salt can be made by using an ion-exchange resin IRA-400 (Cl^-). For the chloride salt: ^{1}H NMR (300 MHz, DMSO-d_6) δ = 9.47 (s, 1H), 8.27 (s, 1H), 7.42−7.34 (m, 5H), 5.28 (s, 2H), 3.11 (s, 6H), 2.84 (s, 3H). For the nitrate salt: ^{1}H NMR (300 MHz, DMSO-d_6) δ = 9.00 (s, 1H), 8.26 (s, 1H), 7.41−7.34 (m, 5H), 5.28 (s, 2H), 3.09 (s, 6H), 2.85 (s, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ = 156.6, 140.3, 136.4, 128.5, 128.3, 77.0, 41.5, 31.7. ESI MS (MeOH): $[M + H]^+$ = 219.80 Da. Anal. Calcd for $C_{12}H_{18}N_4O_4$: C, 51.06; H, 6.43; N, 19.85. Found: C, 51.20; H, 6.95; N, 20.01.

2-(Benzyloxyimino)-*N*′-ethyl-*N*,*N*-dimethylacetimidamide Hydrochloride Salt (14c). To a solution of **13c** (14.2 g, 36.8 mmol) in anhydrous THF (250 mL) cooled to 0−5 °C (ice-bath) was added Me_2NH (2 M in THF, 36.8 mL, 73.6 mmol, 2 equiv). The mixture was stirred at 0−5 °C for 5 min and at rt 40 min and then evaporated. The crude oil was purified by column chromatography (silica gel, CH_2Cl_2→9:1, CH_2Cl_2:MeOH) to give a yellowish oil. **14c** as the triflate salt was dissolved in small amount of hot 5% MeOH in H_2O and passed through an ion-exchange resin column IRA-400 (Cl). The aqueous layer was washed with Et_2O (1 × 100 mL) and evaporated. **14c** (4.4 g, 16.3 mmol) was dissolved in H_2O (50 mL) and a solution of $AgNO_3$ (2.8 g, 16.3 mmol) in H_2O (50 mL) was added. The mixture was stirred at rt exposed to sunlight for 1 h and then filtered, passed through IRA-400 (Cl^-) column, and evaporated to give a yellowish oil that crystallized after drying on high vacuum (4.4 g, 44%). ^{1}H NMR (300 MHz, DMSO-d_6) δ = 9.48 (br s, 1H), 8.30 (s, 1H), 7.42−7.30 (m, 5H), 5.27 (s, 2H), 3.28−3.04 (m, 8H), 0.99 (t, J = 7.2 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ = 155.8, 140.5, 136.6, 128.4, 128.3, 128.2, 76.8, 41.3, 40.0, 15.2. ESI MS for $[M + H]^+$ = 233.88 Da.

2-(Benzyloxyimino)-*N*,*N*-dimethyl-*N*′-propylacetimidamide Hydrochloride Salt (14d). The title compound was obtained similarly to **14c**, but the reaction time was 1 h (yellow oil, 23%). ^{1}H NMR (300 MHz, DMSO-d_6) δ = 9.45 (br s, 1H), 8.30 (s, 1H), 7.42−7.31 (m, 5H), 5.27 (s, 2H), 3.17−3.06 (m, 8H), 1.44−1.33 (m, 2H), 0.71 (t, J = 7.5 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ = 156.0, 140.5, 136.6, 128.4, 128.3, 128.2, 76.8, 46.3, 41.5, 22.8, 10.6. ESI MS for $[M + H]^+$ = 247.88 Da.

2-(Hydroxyimino)-*N*,*N*-dimethylacetimidamide Hydrochloride Salt (15a). To a mixture of **14a** (500 mg, 2.1 mmol) in anhydrous CH_2Cl_2 (10 mL) was added anhydrous $AlCl_3$ (838 mg, 6.3 mmol, 3 equiv). The mixture was stirred at rt for 1 day then MeOH was carefully added followed by H_2O (1 mL). The mixture was evaporated and the yellow residue was purified by column chromatography (silica gel, CH_2Cl_2→8:2, CH_2Cl_2:MeOH) to give a white solid. The product was dissolved in H_2O (2 mL), passed through the IRA-400 (Cl) column, and evaporated to give a white solid (300 mg, 96%), mp = 145.7 °C (dec). ^{1}H NMR (300 MHz, DMSO-d_6) δ = 13.11 (s, 1H), 9.20−8.75 (m, 2H), 8.21 (s, 1H), 3.22 (s, 3H), 3.14 (s, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ = 156.9, 139.0, 40.4, 33.9. ESI MS for $[M + H]^+$ = 115.95 Da.

2-(Hydroxyimino)-*N*,*N*,*N*′-trimethylacetimidamide Hydrochloride Salt (15b). A mixture of **14b** (500 mg, 1.95 mmol) and 10% Pd/C (50% wet, 413 mg, 0.195 mmol, 10% mol) in anhydrous THF (20 mL) was stirred under H_2 atmosphere (balloon) at rt overnight. The reaction was filtered through Celite, washed with *i*-PrOH, and evaporated. The crude product was purified by column chromatography (silica gel, CH_2Cl_2→8:2, CH_2Cl_2:MeOH). Unreacted starting material was recovered (100 mg) and the product was obtained as a yellowish solid (250 mg, 97%), mp = 138.9 °C. ^{1}H NMR (300 MHz, DMSO-d_6) δ = 12.97 (br s, 1H), 9.29 (br s, 1H), 8.07 (s, 1H), 3.15 (s, 6H), 2.93 (s, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ = 157.3, 138.9, 40.6, 31.4. ESI MS for $[M + H]^+$ = 129.7 Da.

N′-Ethyl-2-(hydroxyimino)-*N*,*N*-dimethylacetimidamide Hydrochloride Salt (15c). A mixture of **14c** (700 mg, 2.6 mmol) and 10% Pd/C (50% wet, 276 mg, 0.13 mmol, 5% mol) in anhydrous THF (50 mL) was stirred under a H_2 atmosphere (balloon) at rt overnight. The reaction was filtered through Celite, washed with MeOH, and evaporated. The crude product was purified by column chromatography (silica gel, CH_2Cl_2→8:2, CH_2Cl_2:MeOH) to give a yellow oil which turned into a white solid once stirred in THF (230 mg, 49%; **15c** was obtained in 41% yield when the reaction was carried out in anhydrous EtOH), mp =155.1 °C (dec). ^{1}H NMR (300 MHz, DMSO-d_6) δ = 12.92 (br s, 1H), 9.38 (br s, 1H), 8.09 (s, 1H), 3.34 (q, J = 7.2 Hz, 2H), 3.14 (s, 6H), 1.13 (t, J = 7.2 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ = 156.9, 138.8, 41.3, 39.9, 15.3. ESI MS for $[M + H]^+$ = 143.88 Da. Anal. Calcd for $C_6H_{14}ClN_3O$: C, 40.11; H, 7.85; N, 23.39. Found: C, 40.07; H, 7.80; N, 23.41.

2-(Hydroxyimino)-*N*,*N*-dimethyl-*N*′-propylacetimidamide Hydrochloride Salt (15d). *From **14d**.* A mixture of **14d** (300 mg, 1.06 mmol) and 10% Pd/C (50% wet, 112 mg, 0.05 mmol, 5% mol) in anhydrous EtOH (5 mL) was stirred under a H_2 atmosphere (balloon) at rt for 4 h. Then reaction was filtered through Celite and evaporated. The crude product was purified by column chromatography (silica gel, CH_2Cl_2→8:2, CH_2Cl_2:MeOH) to give a white solid (50 mg, 24%), mp =135.1 °C (dec).

*From **18a** (Two-Step Procedure).* To a solution of **18a** (2.8 g, 10.5 mmol) in $MeNO_2$ (20 mL) at rt was added MeOTf (1.2 mL, 1.7 g, 10.5 mmol). The reaction mixture was stirred at rt overnight and then evaporated. The crude product from the previous step was dissolved in anhydrous THF (100 mL) and Me_2NH (2 M in THF, 10.5 mL, 21 mmol, 2 equiv) was added. The reaction was carried out at rt for 30 min and then evaporated. The crude mixture was purified by column chromatography (silica gel, CH_2Cl_2→9:1, CH_2Cl_2:MeOH) to give **15d** as the triflate salt. The chloride salt was obtained using the IRA-400 (Cl) ion-exchange resin (yellow oil, 880 mg, 43% over two steps). ^{1}H NMR (300 MHz, DMSO-d_6) δ = 12.93 (br s, 1H), 9.42 (br s, 1H), 8.08 (s, 1H), 3.30−3.20 (m, 2H, the signal overlaps with residual H_2O), 3.15 (s, 6H), 1.59−1.47 (m, 2H), 0.82 (t, J = 7.5 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ = 157.0, 138.8, 46.3, 41.5, 22.8, 10.7. ESI MS for $[M + H]^+$ = 157.75 Da.

Ethyl 2-(4-Methoxybenzyloxyimino)acetate (16). A mixture of ethyl glyoxylate (50% in toluene, 75.3 g, 369 mmol) and NH_2OH × HCl (25.6 g, 369 mmol) in CH_3CN:H_2O (9:1, 300 mL) was stirred at rt for 5 min and then Et_3N (51.7 mL, 37.5 g, 369 mmol) was added dropwise. The reaction was carried out at rt for 1 h and then evaporated. The residue was dissolved in H_2O (50 mL) and Et_2O (300 mL). The organic layer was washed with saturated NH_4Cl (50 mL), dried over Na_2SO_4, filtered, and evaporated to give colorless wet crystals of ethyl 2-(hydroxyimino)acetate (41.9 g, 97%). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 9.52 (br s, 1H), 7.56 (s, 1H), 4.32 (q, J = 7.1 Hz, 2H), 1.34 (t, J = 7.1 Hz, 3H).

To a mixture of NaH (60%, 5.64 g, 141.0 mmol, 1.1 equiv) in anhydrous DMF (650 mL) cooled to 0−5 °C was added dropwise a solution of ethyl 2-(hydroxyimino)acetate (15 g, 128.2 mmol) in anhydrous DMF (50 mL). After addition, the cooling bath was removed and the reaction was stirred at rt for 1 h. Finally, a solution of 1-(chloromethyl)-4-methoxybenzene (17.5 mL, 20.1 g, 128.2 mmol) in anhydrous DMF (50 mL) was added and the reaction was stirred at rt overnight. After evaporation, the yellow oil was dissolved in H_2O (100 mL) and Et_2O (500 mL). The organic layer was washed with brine (3 × 50 mL), dried over Na_2SO_4, filtered, and evaporated. The

crude product was purified by column chromatography (silica gel, hexanes→9:1, hexanes:EtOAc) to give a light yellow oil (22 g, 72%). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.51 (s, 1H), 7.34−7.30 (m, 2H), 6.92−6.88 (m, 2H), 5.23 (s, 2H), 4.32 (q, J = 7.2 Hz, 2H), 3.81 (s, 3H), 1.34 (t, J = 7.2 Hz, 3H).

2-(4-Methoxybenzyloxyimino)-*N*-propylacetamide (17a). The title compound was obtained similarly to **11d** (yellowish solid, 89%). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.43 (s, 1H), 7.32−7.27 (m, 2H), 6.93−6.88 (m, 2H), 6.49 (br s, 1H), 5.11 (s, 2H), 3.81 (s, 3H), 3.28 (q, J = 6.9 Hz, 2H), 1.63−1.51 (m, 2H), 0.94 (t, J = 7.5 Hz, 3H).

***N*-Butyl-2-(4-methoxybenzyloxyimino)acetamide (17b).** The title compound was obtained similarly to **11d** but using *n*-BuNH_2 (white solid, 76%). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 7.43 (s, 1H), 7.33−7.27 (m, 2H), 6.94−6.87 (m, 2H), 6.46 (br s, 1H), 5.11 (s, 2H), 3.82 (s, 3H), 3.35−3.28 (m, 2H), 1.58−1.48 (m, 2H), 1.43−1.30 (m, 2H), 0.94 (t, J = 7.2 Hz, 3H).

2-(4-Methoxybenzyloxyimino)-*N*-propylethanethioamide (18a). The title compound was obtained similarly to **12a** (yellow oil, 80%). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.19 (br s, 1H), 7.78 (s, 1H), 7.31−7.27 (m, 2H), 6.93−6.88 (m, 2H), 5.12 (s, 2H), 3.82 (s, 3H), 3.69−3.62 (m, 2H), 1.79−1.67 (m, 2H), 1.00 (t, J = 7.5 Hz, 3H).

***N*-Butyl-2-(4-methoxybenzyloxyimino)ethanethioamide (18b).** The title compound was obtained similarly to **12a** (yellow oil, 97%). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 8.17 (br s, 1H), 7.77 (s, 1H), 7.31−7.27 (m, 2H), 6.93−6.88 (m, 2H), 5.11 (s, 2H), 3.82 (s, 3H), 3.77−3.65 (m, 2H), 1.73−1.63 (m, 2H), 1.48−1.35 (m, 2H), 0.97 (t, J = 7.2 Hz, 3H).

***N*′-Butyl-2-(hydroxyimino)-*N*,*N*-dimethylacetimidamide (19).** The title compound was obtained similarly to **15d** but using **18b** (yellow oil, 79%). ^{1}H NMR (300 MHz, $CDCl_3$) δ = 12.96 (br s, 1H), 9.57 (br s, 1H), 8.08 (s, 1H), 3.30−3.26 (m, 2H, the signal overlaps with residual H_2O), 3.15 (s, 6H), 1.55−1.45 (m, 2H), 1.31−1.18 (m, 2H), 0.84 (t, J = 7.2 Hz, 3H). ^{13}C NMR (125 MHz, DMSO-d_6) δ = 157.0, 138.8, 44.3, 41.4, 31.6, 19.0, 13.5. ESI MS for $[M + H]^+$ = 171.88 Da.

Determination of pK_a. Twenty-two buffers (50 mM, ionic strength = 300 mM, pH from 3 to 13.5) used in these studies were prepared according to the procedures from http://www.bioinformatics.org/JaMBW/5/4/index.html. To a 900 μL buffer solution was added 100 μL of 10 mM solution of **15c** in H_2O. The UV spectrum for each pH point was recorded in the range of 220−700 nm in triplicate, and differences in absorbance at 260 nm were used for the pK_a determination. pK_a values were calculated from the correlation of absorbance/pH using the equation below

$$Y_{obs} = C_1 + \frac{C_2 \times 10^{(pH - pK_1)}}{1 + 10^{(pH - pK_1)}} + \frac{C_3 \times 10^{(pH - pK_2)}}{1 + 10^{(pH - pK_2)}}$$

where C_1 is the absorbance at low pH, C_2 is the increase in absorbance resulting from deprotonation of the functional group with a pK_a of pK_1, and C_2 is the increase in absorbance due to deprotonation of the functional group with a pK_a of pK_2.

Enzyme Studies. Recombinant AChE, β-lactoglobulin from bovine milk (BLG), acetylthiocholine (ATC), butyrylthiocholine (BTC), and 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) were purchased from Sigma Aldrich Chemical Co., (St. Louis, MO). BuChE and ETP were generous gifts from O. Lockridge of the University of Nebraska Medical Center. Syntheses and purification methods of OP model compounds were previously reported.[28,29] Reactivation studies were conducted the same for both AChE and BuChE enzymes. Enzyme was diluted in PBS (pH 7.4) containing 50 ng/μL BLG (PBS/BLG), and a sample was taken and set aside for noninhibited controls. The remaining portion was incubated with a nerve agent model compound or ETP (Figure 7) for sufficient time to achieve 90% or greater inhibition (i.e., ca. 15 min). The reaction of the enzyme with nerve agent model compounds afforded covalent modification of the enzyme identical to that obtained with authentic nerve agents.[28,29] An excess of OP was removed from inhibited enzyme by filtration through a 10 kDa MWCO filter with a modified PES membrane (VWR Scientific, Inc., San Diego, CA), followed by two washes prior to the final resuspension in PBS/BLG (pH 7.4). An excess of 1000-fold dilution of OP was achieved. Enzyme was then added to PBS/BLG (pH 7.4) containing amidine−oxime, MINA, or 2-PAM (100 μM oxime with 2% DMSO after addition of enzyme) or vehicle. Oxime samples were equilibrated at 37 °C prior to addition of enzyme. Enzyme was allowed to reactivate at 37 °C for 20 min (BuChE) or 1 h (AChE), at which time the catalytic activity was determined using a modified Ellman's assay[36] in the presence of 1 mM substrate in PBS/BLG (pH 7.4). The Ellman assay involved a 20-fold dilution of enzyme from the oxime incubation, yielding incubation concentrations in the range of 10−50 units L^{-1} (where 1 unit cleaves 1 μmol of substrate per min in PBS pH 7.4, room temperature) and 5 μM oxime. Two reactivation samples were prepared per oxime. The reported data show the average esterase activity for each oxime divided by the average activity of noninhibited control samples and the propagated errors of the average activities.

In Vivo Studies. Adult Swiss Webster female mice (from Taconic) were housed in groups of four and maintained in a temperature-controlled environment on a 12 h:12 h light cycle (0600 h on, 1800 h off) upon arrival to the laboratory. Animals were given free access to food and water during a 1-week habituation period to the laboratory. Animals used in the research studies were handled, housed, and killed in accordance with current IACUC protocol (024−2008) and NIH guidelines regarding the use and care of laboratory animals and all applicable local, state, and federal regulations and guidelines.

***rac*-GDC Studies.** Separate groups of mice were amidine−oximes (145 μmol/kg), 2-PAM (145 μmol/kg), or vehicle pretreated (ip) 30 min prior to ***rac*-GDC** (0.25 mg/kg in 0.25 mL/mouse ip). Each test compound and 2-PAM as hydrochloride salts were dissolved in isotonic saline and administered to separate groups in a volume of 0.25 mL/mouse. The survival was recorded after 24 h from the onset of the experiment. Animals that survived the 24 h period were then immediately killed by cervical dislocation.

***Sp*-GB-Am Studies.** At the 0 min time point, mice received ***Sp*-GB-Am** hydrochloride (0.08 mg/kg in 0.25 mL/mouse in saline, ip). Five minutes later amidine−oxime **15d** (145 μmol/kg), MINA (145 μmol/kg), or vehicle was administered ip in isotonic saline (0.25 mL/mouse). The survival was recorded after 24 h. Animals that survived 24 h were then immediately killed by cervical dislocation.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Tables with compounds purity data, binding affinity to AChE and BuChE for **19**, and reactivation of OP-inhibited AChE using **15a**−**d**, **19**, 2-PAM, and MINA after 20 min. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

ACh, acetylcholine; AChE, acetylcholinesterase; ATC, acetylthiocholine; BBB, blood−brain barrier; BuChE, butyrylcholinesterase; BTC, butyrylthiocholine; CNS, central nervous system; ChE, cholinesterase enzymes; DAM, diacetylmonooxime; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; BLG, β-lactoglobulin; PMB, *p*-methoxybenzyl protecting group; MeOTf, methyl trifluoromethanesulfonate; MINA, monoisonitrosoacetone; OP, organophosphate; 2-PAM, pralidoxime

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