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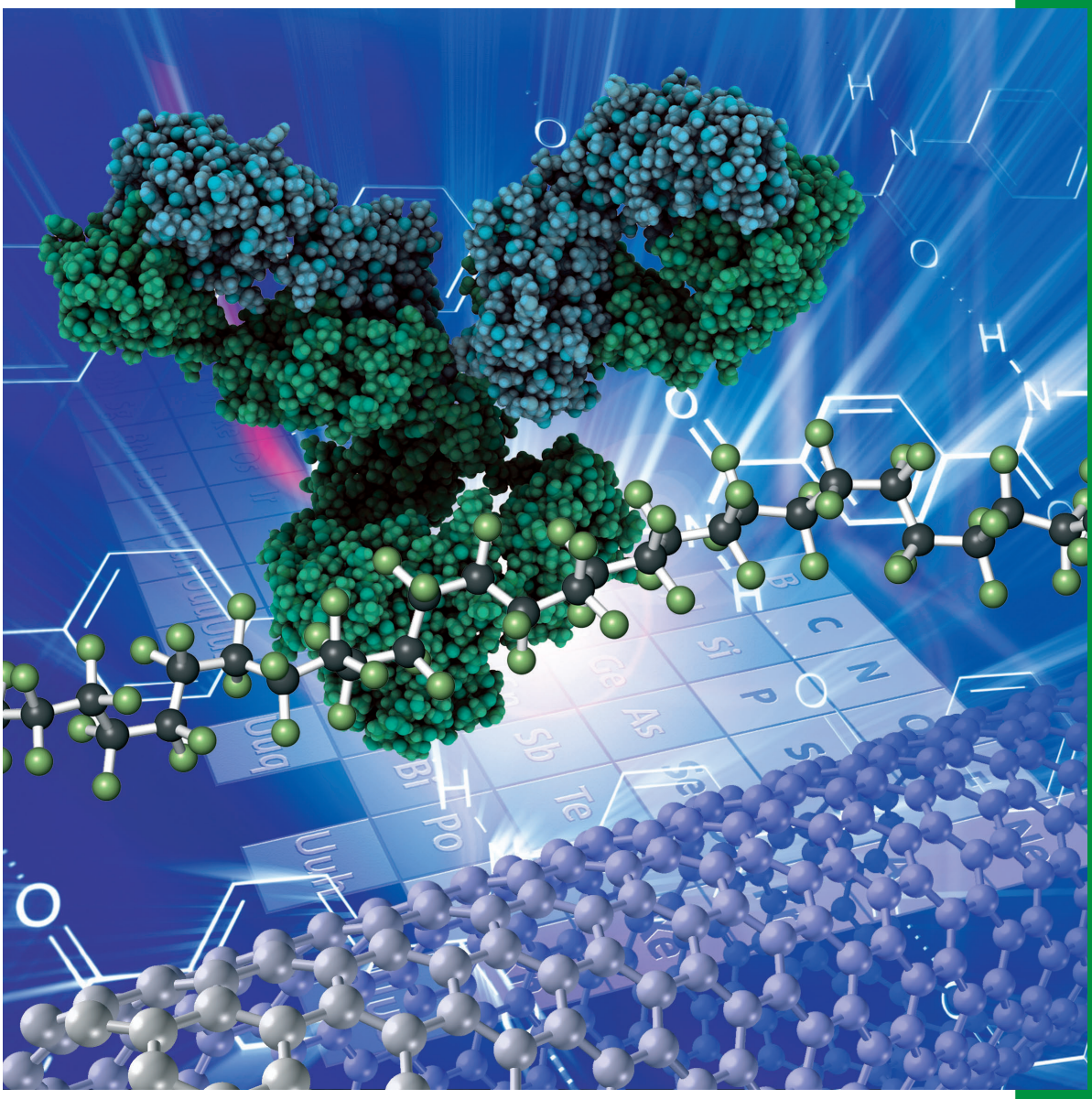

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β-Aminoalcohols as Potential Reactivators of Aged Sarin-/Soman-Inhibited Acetylcholinesterase

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Organophosphate nerve agents inhibit the enzyme acetylcholinesterase (AChE), which is involved in nerve signal transduction, by forming covalent adducts with its catalytic serine residue. AChE adducts with soman and sarin nerve agents undergo dealkylation, a process known as aging, within a few minutes and a few hours, respectively. This transformation is detrimental because it precludes reactivation of AChE with known oxime-based antidotes. Here, we designed a β-aminoalcohol molecule for aged AChE reactivation, using a multi-tiered computational approach. This approach includes high-quality quantum mechanical/molecular mechanical calculations, providing reliable reactivation steps and energetics. The calculations suggest that the designed β-aminoalcohol can selectively reactivate aged sarin-/soman-inhibited AChE. Furthermore, unlike existing antidotes, the designed β-aminoalcohol lacks a permanent charge, making it potentially active in the central nervous system. The mechanistic insights of this study can help guide the development of new AChE reactivators with improved access to the central nervous system.

Introduction

Nerve agents disrupt nerve signal transduction by covalently inhibiting acetylcholinesterase (AChE), an enzyme that breaks up the neurotransmitter acetylcholine in neural and neuromuscular junctions. Unless treated promptly with an antidote that includes cholinergic receptor antagonists (e.g., atropine), inhibitors of acetylcholine release (e.g., diazepam), and oxime-based AChE reactivators (e.g., 2-pralidoxime [2-PAM]; Figure 1),[1] the developing cholinergic crises can cause neurological damage and muscle function loss, which ultimately lead to death.

The nerve agents soman and sarin present additional challenges because of their ability to undergo dealkylation after forming covalent adducts with the catalytic serine residue of AChE. Soman is of particular concern because the adduct it forms with AChE ages within a few minutes, whereas the sarin adduct ages within a few hours. Serendipitously, the aged AChE adducts of soman and sarin are identical.[2] Unlike its precursors, the aged AChE adduct is inert to existing oxime antidotes.[2] This property dramatically enhances the toxicity of soman and sarin.[1a]

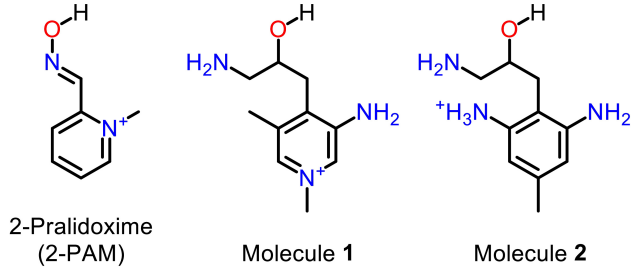

Figure 1. An approved oxime antidote, 2-pralidoxime (2-PAM), and two β-aminoalcohol molecules (*molecules 1* and *2*) considered in the present study as reactivators of the aged sarin-/soman-inhibited acetylcholinesterase (AChE) adduct.

Although existing oxime antidotes can reactivate adducts of AChE and nerve agents within a narrow window of time between exposure and aging by dealkylation, their therapeutic effects are limited to neural and neuromuscular junctions of the peripheral nervous system. These antidotes are not as effective at neural junctions of the central nervous system[1b,c] because their permanent positive charge, which mimics that of acetylcholine and aids in binding the active site of AChE, prevents them from crossing the blood-brain barrier and thereby enabling therapeutic interventions in the central nervous system.[1b]

One way to aid the permanently charged oxime antidotes in crossing the blood-brain barrier is to conjugate them with other small molecules that are actively transported across the barrier.[3] Alternatively, new antidotes could be developed without the permanent charge so that they can passively cross cell membranes in the blood-brain barrier but then become

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positively charged upon protonation—a condition required for AChE binding and reactivation.[1c,4]

Once aging occurs, existing antidotes no longer help reactivate AChE. In the absence of reactivators of the aged AChE adduct, restoring the concentration of active AChE to normal levels by *de novo* biosynthesis would require several days, which is inadequate for acute poisoning scenarios.[5] Therefore, finding a way to rapidly reactivate aged AChE in situ would significantly reduce the adverse effects of nerve agent poisoning and help save human lives.

We are not aware of any antidote that selectively reactivates aged AChE by itself in either the peripheral or central nervous system. Unfortunately, neither oximes (aldoximes) nor amidoximes, which are most successful in reactivating AChE in the peripheral nervous system before aging[1c,4c, 6], can reactivate the aged AChE adduct.

Because the aged AChE adduct is inert to existing oxime antidotes, a strategy has been proposed whereby the aged adduct is first alkylated and then subjected to reactivation with oximes.[7] However, finding a nontoxic alkylating agent that selectively alkylates the aged AChE adduct under physiological conditions has proven difficult.[7] Furthermore, using an alkylating agent as a prerequisite to the oxime reactivation may be problematic for pharmacokinetic reasons.

An ideal reactivation strategy, in our opinion, would be to use a small molecule that selectively reactivates aged AChE without the need for pretreatment with alkylating agents. In addition, the ideal candidate molecule should cross the blood-brain barrier, which is impenetrable to many permanently charged molecules. Finding an antidote capable of reactivating the aged AChE adduct and crossing the blood-brain barrier to deliver therapeutic effects in both the peripheral and central nervous systems was the ultimate goal of the present study.

Results and Discussion

Considerations for designing aged AChE reactivators

Several factors may preclude reactivation of the aged AChE adduct by oxime-based antidotes. The main challenge is the permanent negative charge, created upon dealkylation, on the phosphonyl moiety of the nerve agents covalently attached to the catalytic serine residue.[2] This negative charge disables the catalytic machinery of AChE by forming a strong salt bridge with the positively charged catalytic histidine residue (H440) and, thereby prevents proton transfer from the histidine residue to the Oγ of the phosphonylated serine residue. Furthermore, the negative charge on the phosphonyl moiety could electrostatically repel the negatively charged nucleophilic oxygen of the oxime antidotes.

Therefore, our design criteria excluded oxime nucleophiles. Instead, we pursued nucleophiles that are initially neutral but attain their full nucleophilic potential at or near the transition state for the Sn2 nucleophilic substitution reaction during reactivation of the aged AChE adduct.

Mechanistic insights from a functionally similar enzyme

Recently, we reported the results of a mechanistic study of an unusual enzyme, CapD, from the superfamily of N-terminal nucleophile (Ntn) hydrolases, which are functionally similar to AChE and serine proteases.[8] These enzymes are also inhibited by phosphonylation.[9] Their active sites, just like the active site of AChE, have an oxyanion hole that helps stabilize the tetrahedral intermediate formed during the Sn2 nucleophilic attack on their natural substrates. The principal difference among CapD, AChE, and serine proteases is that instead of a canonical catalytic triad, such as Asp-His-Ser, the CapD enzyme uses a single N-terminal threonine residue. The key moiety that enables the catalytic function of the threonine residue is β-aminoalcohol.

Our earlier calculations demonstrated that the nucleophilic OγH group of the β-aminoalcohol moiety from the N-terminal catalytic threonine of CapD is deprotonated during the nucleophilic attack on its substrate, transferring its proton to the originally neutral N-terminal amino group.[10] Unlike oximes, the β-aminoalcohol nucleophile is neutral at the beginning of the nucleophilic attack but develops a negative charge during an intramolecular proton transfer. Therefore, β-aminoalcohol satisfies our design criteria for having an uncharged nucleophile.

Incorporating mechanistic insights into the design of small molecule reactivators

The knowledge of the mechanism of CapD catalysis inspired us to design β-aminoalcohol molecules that would bring the CapD-like catalytic machinery into the active site of aged AChE. Therefore, we derived a number of small molecules by grafting the nucleophilic β-aminoalcohol fragment of the catalytic threonine residue of CapD onto the anchoring pyridine ring of the currently approved 2-PAM antidote (Figure 1) and several of its variants. The pyridine ring of 2-PAM has a permanent positive charge that should help anchor the grafted β-aminoalcohol in the active site of the aged AChE adduct. However, this permanent charge would prevent the molecule from crossing the blood-brain barrier.

Testing the designed small molecule reactivators

To test whether the designed molecules could reactivate aged AChE, we used a multi-tiered computational chemistry approach comprising *1*) docking[11] of a molecule into the active site of the aged AChE adduct, *2*) molecular dynamic simulations of the complex in solution,[12] and *3*) hybrid quantum mechanical/molecular mechanical (QM/MM)[13] reaction path[8,10] calculations to assess the steps and energetics of aged AChE reactivation. Details of the approach are provided in the Supporting Information.

The final stage of the multi-tiered simulations involved computationally intensive QM/MM simulations, using density functional theory for the QM portion of the simulations. This kind of QM/MM simulation allows high-fidelity studies of

chemical reactions in enzymes and has been extensively used to study AChE catalysis,[14] phosphonylation,[15] aging,[16] and reactivation with oxime antidotes before aging.[16b,17] To our best knowledge, this work is the first such QM/MM study of reactivation of the aged AChE adduct. Furthermore, the QM region in our QM/MM study is one of the largest reported to date (Figure 2). The QM region included a catalytic triad (E327, H440, and S200), an oxyanion hole (G118 and G119), a choline anchoring residue (E199), a nearby Y121, five water molecules (w1-w5), and a β-aminoalcohol ligand (*molecule 1* or *2*).

Aged AChE reactivation with molecule 1

One of the best molecules identified that satisfied our design criteria was *molecule 1* (Figure 1). This molecule has an asymmetric carbon, with the *S*-enantiomer having the best configuration for reactivation of the aged AChE adduct. Despite the lack of a negative charge, the β-aminoalcohol nucleophile grafted on the positively charged pyridine anchor exhibited a reactivation barrier in excess of 50 kcal·mol^{-1}. The energy of the final product bound to reactivated AChE was also very high (20 kcal·mol^{-1}).

The highly unfavorable reactivation with *molecule 1* proceeded in one step. The absence of the expected trigonal bipyramidal metastable intermediate was most likely due to the requirement that it bear a doubly negative charge and, thus, would not be sufficiently stabilized by the electrostatic environment of the aged AChE adduct. The water molecules in the active site were coupled to the reactivation and rearranged before the nucleophilic attack. Given the high barrier and endothermicity of aged AChE reactivation with *molecule 1*, this reaction is unlikely to occur under physiological conditions.

Analysis of the optimized reaction path for *molecule 1* (not shown) demonstrated that the salt bridge between the catalytic H440 and the phosphonyl strongly resisted transfer of the proton from H440 to the Oγ oxygen of the phosphonylated S200, preventing reactivation of aged AChE. Developing the second negative charge on the phosphonyl during the nucleophilic attack strengthened the salt bridge between H440 and the phosphonyl attached to S200. This observation suggested that the strength of the salt bridge would need to be reduced to achieve aged AChE reactivation.

Aged AChE reactivation with molecule 2

To reduce the strength of the salt bridge between the catalytic H440 and the aged phosphonyl, we incorporated an analogue of histidine into β-aminoalcohol *molecule 2* (Figure 1). *Molecule 2* was derived from *molecule 1* by swapping the C atom of the methyl group attached to the pyridine ring and the N atom of the tertiary amine of the pyridine to create a positively charged NH_3^+ group in the vicinity of both the choline anchoring E199 and the phosphonyl oxygen of the covalently inhibited catalytic S200.

Swapping these atoms also moved the positive charge from the pyridine ring to its periphery in *molecule 2*. Unlike *molecule 1*, which has a permanent positive charge on the

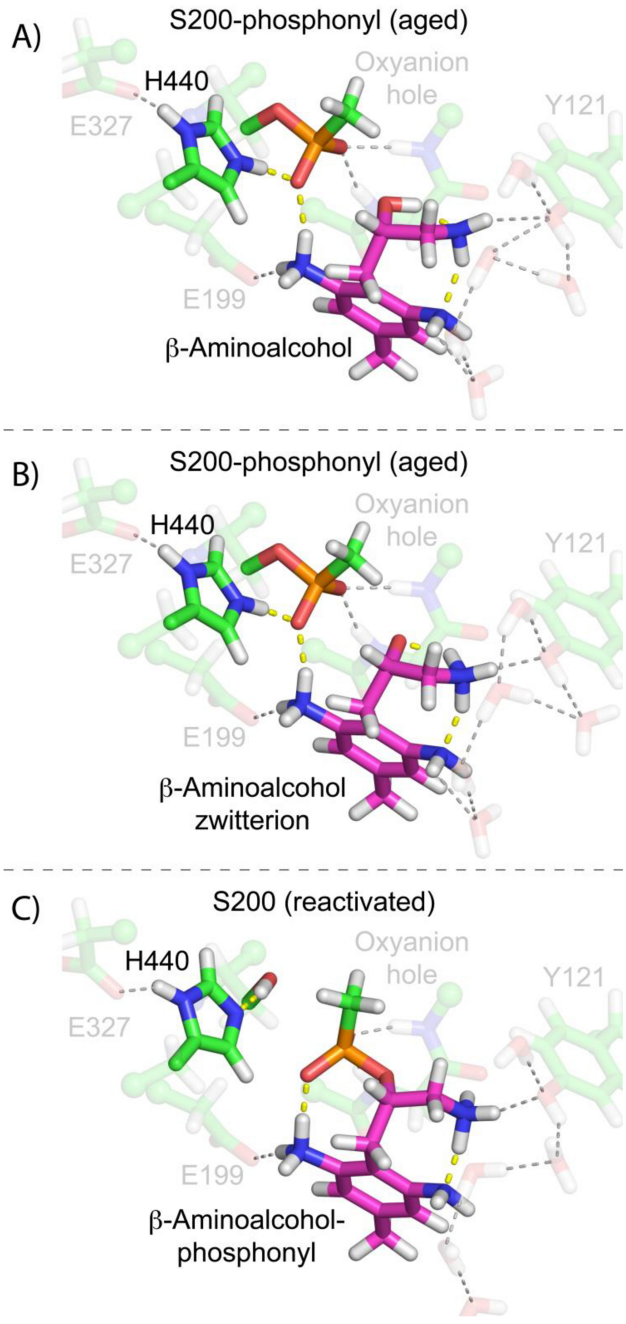

Figure 2. Representative structures along the reaction path for reactivation of aged sarin-/soman-inhibited AChE adduct with β-aminoalcohol *molecule 2*. *A*) Complex of aged AChE with *molecule 2*. *B*) Metastable zwitterionic intermediate of β-aminoalcohol *molecule 2*. *C*) Reactivated AChE bound to the adduct of β-aminoalcohol *molecule 2* with the aged phosphonyl moiety of sarin/soman. Only atoms that are included in the quantum mechanical (QM) region of the QM/molecular mechanical calculation are shown. Carbon atoms of the aged AChE adduct are depicted in green. Carbon atoms of β-aminoalcohol *molecule 2* are shown in magenta. Hydrogen, nitrogen, and oxygen atoms are white, blue, and red, respectively. Peripheral hydrogen bonds are shown with dashed gray lines, whereas those of particular relevance for reactivation are shown with yellow dashed lines. Residues of the active site not directly involved in the reaction are shown in faded, transparent colors. Atoms that link the QM region to the rest of the system described by molecular mechanics are depicted with transparent spheres around them. These atoms are substituted for hydrogen atoms, and their bond lengths are adjusted accordingly during QM calculations.

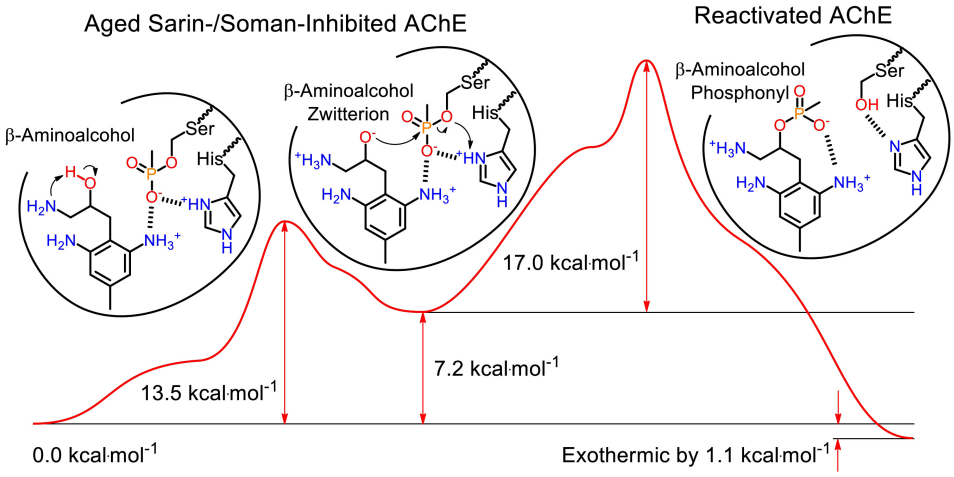

Figure 3. Steps and energetics of aged sarin-/soman-inhibited AChE reactivation with β-aminoalcohol *molecule 2*.

anchoring pyridine ring, *molecule 2* is positively charged because of ionization of one of its amino groups. Importantly, this charged group could become neutral under certain conditions that would enable *molecule 2* to spontaneously cross the blood-brain barrier and therefore potentially act in the central nervous system. This property, which is required to deliver therapeutic effects to the central nervous system, makes *molecule 2* our ideal candidate.

The positively charged amino group of *molecule 2* formed a second salt bridge with the phosphonyl (Figure 2 *A*), allowing the catalytic histidine residue to transfer its proton to the oxygen of the phosphonylated serine residue. Optimization of the reaction paths for aged AChE reactivation with *molecule 2* yielded a reactivated AChE with significantly lower energy than did that with *molecule 1*. Reactivation with *molecule 2* was slightly exothermic, by 1.1 kcal·mol^{-1} (Figure 3). Furthermore, the optimized reaction paths revealed a metastable intermediate (Figure 3). The intermediate was formed by an intramolecular proton transfer within the nucleophilic β-aminoalcohol group of *molecule 2*, which created a zwitterionic β-aminoalcohol group with a negatively charged oxygen for the nucleophilic attack and a positively charged β-NH_3^+ group (Figure 2*B*). This metastable intermediate had energy of 7.2 kcal·mol^{-1} (Figure 3). The barrier for the formation of this intermediate was 13.5 kcal·mol^{-1}.

The nucleophilic attack from the intermediate with the zwitterionic β-aminoalcohol group proceeded by transfer of the phosphonyl moiety from the inhibited catalytic S200 to the zwitterionic β-aminoalcohol group, with a barrier of 17.0 kcal·mol^{-1}. This attack successfully reactivated aged AChE (Figures 2*C* and 3). Although the reaction path did not reveal a stable trigonal bipyramidal intermediate, it did have a shoulder in the potential energy profile with the corresponding structure. In the final stages of the reaction, the five water molecules reorganized, providing additional stabilization to the reactivated AChE complex.

Importantly, the reverse reaction, which could re-inhibit AChE, had a barrier of 25.3 kcal·mol^{-1}; hence, it should be substantially less likely than reactivation. This prediction suggests that aged phosphonylated *molecule 2* would be unable to re-inhibit AChE.

The success of *molecule 2* will depend on its ability to achieve the protonation state used for the reactivation reaction calculations upon binding aged AChE. Using simple pKa prediction tools[18] to estimate the pKas of the three ionizable amino groups of *molecule 2* in solution, we find that the β-amine is protonated first with a pKa of 9.4, after which the two equivalent amines on the benzene ring are protonated second and third with pKas of 5.1 and 2.8, respectively. Therefore, under physiological conditions, the majority of molecules in a solution of *molecule 2* will be singly protonated at the β-amine. However, proteins shift solution pKas by as much as 8 pKa units.[19] Furthermore, these predictions do not take into account intramolecular interactions such as the one seen between the β-amine and the unprotonated amine of the benzene ring (Figure 2).

The solution pKa of the β-amino group of *molecule 2* can be altered significantly by using the lessons learned from Ntn hydrolases. In the Supporting Information, we provide the results of several such pKa calculations for a number of derivatives of *molecule 2*. Furthermore, the methyl group of the benzene ring helps increase the pKas of the two amino groups on the ring (see Supporting Information).

Using available experimental kinetic data[20] and estimates of the free energy of activation in the two-step catalytic mechanism of AChE,[21] we estimated the pre-exponential factor for the acylation and deacylation steps of S200 to be on the order of 6×10^{12} s^{-1}. Given the predicted reactivation barrier of 17.0 kcal·mol^{-1} and assuming three orders of magnitude reduction of the pre-exponential factor while excluding unknown entropic contributions to the activation free energy, we estimate the reaction rate for aged AChE reactivation with *molecule 2* to be on the order of 2.6×10^{-3} s^{-1} at 300 K. Including the entropic contributions may significantly improve this estimate. This estimated reaction rate would amount to a 50% reactivation time of approximately 4 minutes.

It remains to be seen whether *molecule 2* will have a therapeutic effect against soman. To estimate the efficiency of aged AChE reactivation with *molecule 2*, one would need to

know the binding constants for *molecule 2* and for its aged phosphonylated product. Nevertheless, the rough estimate of the aged AChE reactivation rate suggests that *molecule 2* may achieve a therapeutic effect against nerve agent poisoning with soman.

In summary, aged AChE reactivation with *molecule 2* proceeded in two consecutive steps and was slightly exothermic (by 1.1 kcal·mol^{-1}).

Conclusions

Our calculations predict that *molecule 2*, unlike its constitutional isomer *molecule 1*, may be able to reactivate aged AChE. The aged phosphonylated *molecule 2* is not expected to re-inhibit AChE. To our best knowledge, *molecule 2* is the first molecule that has been demonstrated, albeit computationally, to reactivate aged AChE without the need for alkylating agents. Our calculations assume that one of the two *molecule 2* amino groups attached to the phenyl ring is protonated. The protonated amino group bridges the choline anchoring negatively charged E199 and the negatively charged phosphonyl of the aged AChE adduct. This group is strategically placed and allows catalytic H440 to transfer its proton to phosphonylated S200.

We wish to emphasize that in addition to the energetics that favor *molecule 2* over *molecule 1*, *molecule 2* differs from *molecule 1* in one important regard. Specifically, *molecule 2* does not have a permanent charge on its anchoring ring and can therefore cross the blood-brain barrier. In contrast, *molecule 1* has a permanent positive charge on its anchoring ring that would prevent it from passively crossing the blood-brain barrier. Therefore, *molecule 2* is expected to act in both the peripheral and central nervous systems and is the ideal candidate for aged AChE reactivation.

Implications of the study for future work

The mechanistic insights gleaned from this study may enable redesign of existing oximes and afford new oximes capable of reactivating aged and nonaged AChE adducts in the central nervous system. Furthermore, because β-aminoalcohols are predicted to successfully reactivate aged AChE, a task considered to be impossible for existing oxime antidotes, we anticipate that β-aminoalcohols may also be able to reactivate nonaged adducts of AChE and nerve agents. These predictions and molecular design strategies are made possible through our multi-tiered computational study of the steps and energetics of aged AChE reactivation.

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Conflict of Interest

The authors declare no conflict of interest.

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