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Characterization of SWRI 80A Reactivation of Recombinant Human Acetylcholinesterase and Identification of Its Absolute Chirality Using Specific Rotation Calculations in Gaussian/16 at the Hartree-Fock Level of Theory

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14. ABSTRACT

The United States Army Medical Research Institute of Chemical Defense (USAMRICD) in collaboration with the Southwest Research Institute (SwRI) has identified an uncharged reactivator of nerve agent-inhibited recombinant human acetylcholinesterase (rHuAChE) that was able to reactivate rHuAChE inhibited by GF (cyclosarin) by 90% within 5 minutes at a concentration of 500 µM during in vitro testing. The oxime reactivator SwRI_80A (80A) was synthesized by SwRI and tested at the USAMRICD. 80A is a racemic mixture of isomers about a chiral carbon atom in a hexyl ring. Each isomer has two E-Z enantiomers about a carbon-nitrogen double bond near the reactivating oxygen on the oxime arm of the molecule. To evaluate the reaction kinetics of each isomer, the racemic mixture was separated on a super-critical-fluid chromatography system. Two peaks were identified and named by the elution order (P1 and P2). This report describes the identification of the chirality of those two peaks.

15. SUBJECT TERMS

Sarin; cyclosarin; acetylcholinesterase; reactivation; oxime chirality

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Abstract

The United States Army Medical Research Institute of Chemical Defense (USAMRICD) in collaboration with the Southwest Research Institute (SwRI) has identified an uncharged reactivator of nerve agent-inhibited recombinant human acetylcholinesterase (rHuAChE) that was able to reactivate rHuAChE inhibited by GF (cyclosarin) by 90% within 5 minutes at a concentration of 500 µM during *in vitro* testing. The oxime reactivator SwRI_80A (80A) was synthesized by SwRI and tested at the USAMRICD. 80A is a racemic mixture of isomers about a chiral carbon atom in a hexyl ring. Each isomer has two E-Z enantiomers about a carbon-nitrogen double bond near the reactivating oxygen on the oxime arm of the molecule. To evaluate the reaction kinetics of each isomer, the racemic mixture was separated on a super-critical-fluid chromatography system. Two peaks were identified and named by the elution order (P1 and P2). The peaks were collected in methanol, partially dried, re-suspended in phosphate buffer (pH 7.4) and tested for the ability to reactivate GF-inhibited rHuAChE at a concentration of 5 µM with 1.67% methanol. Peak 1 was found to be responsible for nearly all of the reactivation when tested at 5 µM for 30 minutes. A second, larger batch of racemic oxime was synthesized by SwRI, was separated into isomeric peaks by two different companies, and was tested at higher concentrations. Optical activity of the isomers was determined via plane polarized light, and isomers were assigned the dextrorotary (+) or levorotary (-) designators by SwRI. Higher concentration testing of the oximes revealed that both isomers could reactivate the inhibited protein. The (+) isomer was initially faster, but reactivation slowed with time. The (-) isomer gave a slower initial reactivation, but continued for longer times. A decision was made to attempt chiral synthesis of the more active isomer, but the absolute chirality of that isomer needed to be identified.

This paper reports the determination of the absolute chirality of the isomers using quantum calculation methods on the DoD High Performance Computing (HPC) clusters in conjunction with polarimetry measurements taken at the USAMRICD. Each chiral molecule was built mathematically, and all unique conformers were determined. Quantum computational methods in Gaussian 16 (ref 1) were used to predict the specific rotation for each conformer. The specific rotations of the conformers for an isomer were combined in an average weighted by the proportion of each conformer in a methanol solution. The computed specific rotation for each isomer was compared to the empirically measured values to reveal the absolute chirality of the (+) and (-) peaks to be the S and R isomers respectively.

BACKGROUND and INTRODUCTION

At cholinergic synapses, acetylcholine (Ach) is the neurotransmitter. Its release from vesicles at the nerve terminal is in response to a depolarization wave at the nerve terminal that results in fusion of Ach-filled vesicles with the synaptic membrane and a massive release of Ach into and across the synapse where Ach receptors are activated in the membrane of the downstream nerve (or muscle fiber), causing a depolarization wave in that downstream cell. The massive release of Ach into the synapse is almost immediately eliminated by the action of the protein acetylcholinesterase (AChE) which is fast enough to complete the elimination of Ach before the upstream nerve fires again and before the downstream nerve has repolarized. The orientation of Ach in the active site of AChE is shown in figure 1, along with that for GF.

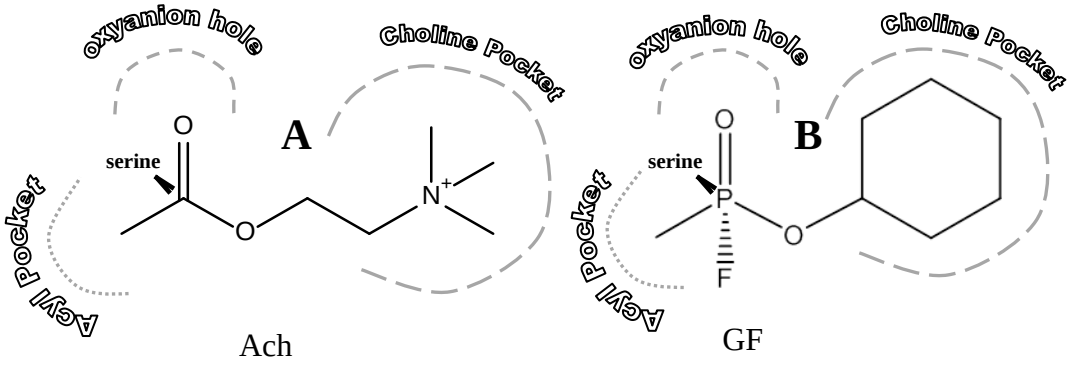


Figure 1. Acetylcholine (left) and the nerve agent GF (right) are shown as they sit in the AChE enzyme. For Ach, the double-bonded oxygen fits into the "oxyanion hole," the leftmost methyl group fits into the "acyl pocket" and the rightmost choline portion fits into the "choline pocket" of the enzyme. The catalytic serine sidechain is just behind the carbonyl atom shown in Ach. The carbonyl carbon makes a covalent bond to the catalytic serine of the enzyme, forming a tetrahedral configuration. This serine bond breaks when a water molecule attacks the carbonyl atom and contributes a proton and a hydroxyl group, forming acetate and choline. Similarly for GF, the double-bonded oxygen fits into the "oxyanion hole," the leftmost methyl group fits into the "acyl pocket" and the rightmost cyclohexyl portion fits into the "choline pocket" of the enzyme (for GB, it is an isopropyl group that fits into this pocket). The catalytic serine sidechain is just behind the phosphonyl atom shown in GF. The phosphorus atom makes a covalent bond to the catalytic serine on AChE, forming a trigonal bipyramidal intermediate that loses the fluorine atom and becomes a tetrahedral configuration. The serine-phosphorus bond does not spontaneously break under the action of a water molecule on the P atom. A stronger attack on the P atom (such as from an oxime) is required to pull the agent moiety from the serine.

The carbonyl carbon of Ach binds covalently to the AChE protein at the active site serine to form a tetrahedral intermediate. During reactivation, a water molecule attacks the carbonyl carbon, the covalent bond breaks and the Ach breaks down into choline and acetate, freeing the enzyme to catalyze the breakdown of another Ach molecule. The phosphorus atom in organophosphorus (OP) nerve agents also covalently binds to the active site serine, loses a "leaving group" (either fluorine or cyanide for G agents; a sulfur containing group for V agents), and forms a tetrahedral configuration. Unlike Ach, this tetrahedral intermediate does not break

down when a water molecule attacks the P atom. A stronger attack on the OP tetrahedral structure is required to break the OP-serine bond, complete the OP breakdown and free the enzyme. Oximes are one class of compounds that have been shown to facilitate the OP breakdown. Many oximes have been tested for reactivation of OP-inhibited enzyme, but the most effective ones are charged compounds. As such, they can ameliorate OP intoxication in most body compartments, but because charged molecules will not cross the blood-brain barrier without assistance, they are less useful in the brain. The Southwest Research Institute (SwRI) in cooperation with the USAMRICD began a QSAR study in 2008 to test reactivation of OP-inhibited acetylcholinesterase by a series of uncharged oximes. The overall results have been reported elsewhere[ref 2], but one oxime, SwRI_80A (80A), stood out as particularly effective during *in vitro* testing for its ability to reactivate GF-inhibited rHuAChE. At a concentration of 18.75 µM (bold red lines in Figure 2 panels A and B) it was found to reactivate the GF-inhibited enzyme by 55% within 6 minutes. At 500 µM, reactivation at 6 minutes was almost complete. This oxime was chosen for further study.

METHODS

Preparation of inhibited and control enzyme

The GF-inhibited acetylcholinesterase was prepared by mixing 6 µL of 3.39 mM GF and 70 µL of 26.5 µM rHuAChE into 391 µL of phosphate buffer (pH 7.4). Inhibition of the enzyme was allowed to proceed for one hour, and the mixture was cleaned on a size-exclusion column to obtain agent-free inhibited enzyme at 4 µM. Enough enzyme was prepared to produce multiple 17 µL aliquots of GF-inhibited enzyme for use in the experiments. Seventeen µL aliquots of the uninhibited (control) enzyme were also prepared using 397 µL of buffer. All control and agent aliquots were stored at -80^{o}C until tested. Before the reactivation experiment, the aliquots were thawed, and 15 µL of control or inhibited enzyme was added to 2 mL of bovine serum albumin (BSA) in pH buffer resulting in an enzyme concentration of 29.78 nM. The BSA is used to stabilize the enzyme during the testing period. The enzyme degrades by about 50% in two hours.

Reactivation of Inhibited Enzyme

A Biomek NFx fluid transport handler with a 96-well pipette head in our reactivation testing facility (the robot) was used for simultaneous 96-well transfers of liquid between the plates. A set of grippers allowed the robot to move and stack plates and to move a plate to/from the input drawer of a Biomek DTX-880 spectrophotometer. The computer controlling the robot also interfaced with and controlled the spectrophotometer so that absorbance files could be saved in real time. At the start of a reactivation measurement, a 96-well transfer of 170 µL of reactivator (at various concentrations) was mixed into the wells of a 96-well reactivation plate containing 170 µL of control or inhibited acetylcholinesterase to start the reactivation reaction. At fixed time points after mixing, 30 µL microliter samples of the reactivating liquid were diluted by a factor of 50 into wells of a substrate plate containing 1.5 mM acetylthiocholine and 1mM 5,5'-dithiobis-(2-nitrobenzoic acid). The final concentration of the enzyme in the substrate plate was 3 nM. A spectrophotometer was used to measure the rate of color development (at 412 Å) as indicative of the enzyme activity at the sampled time. The ratio of the inhibited enzyme activity to the corresponding control enzyme activity was used to determine the percentage of reactivation at that time/concentration for each well.

RESULTS

Reactivation of GF-inhibited enzyme by racemic 80A

The racemic 80A compound was tested *in vitro* at the USAMRICD at a number of concentrations from 0 µM to 1000 µM against GF-inhibited human acetylcholinesterase from Sigma Aldrich or against recombinant human acetylcholinesterase from Alitropic Tech. The racemic 80A (based on a "flattened" structure of Huperzine-A) was found to inhibit control enzyme with a computed K_I of (1108±166) µM (R^2 = 0.8365). Reactivation after 5 minutes was nearly (84%) complete for the 500 µM concentration and 55% complete for the 18.75 µM concentration. From the reactivation data observed over a range of concentrations, the bimolecular rate constant was computed to be (1.535±0.48) $mM^{-1}min^{-1}$ (R^2 = 0.9886). Figure 2a shows the data at different time scales (panels A and B) along with the exponential association curve fits, the control enzyme inhibition (panel C) and the initial slope of the spectrophotometric readings at each time as a function of concentration (panel D). Figure 2b shows the same results for a lower range of oxime concentrations.

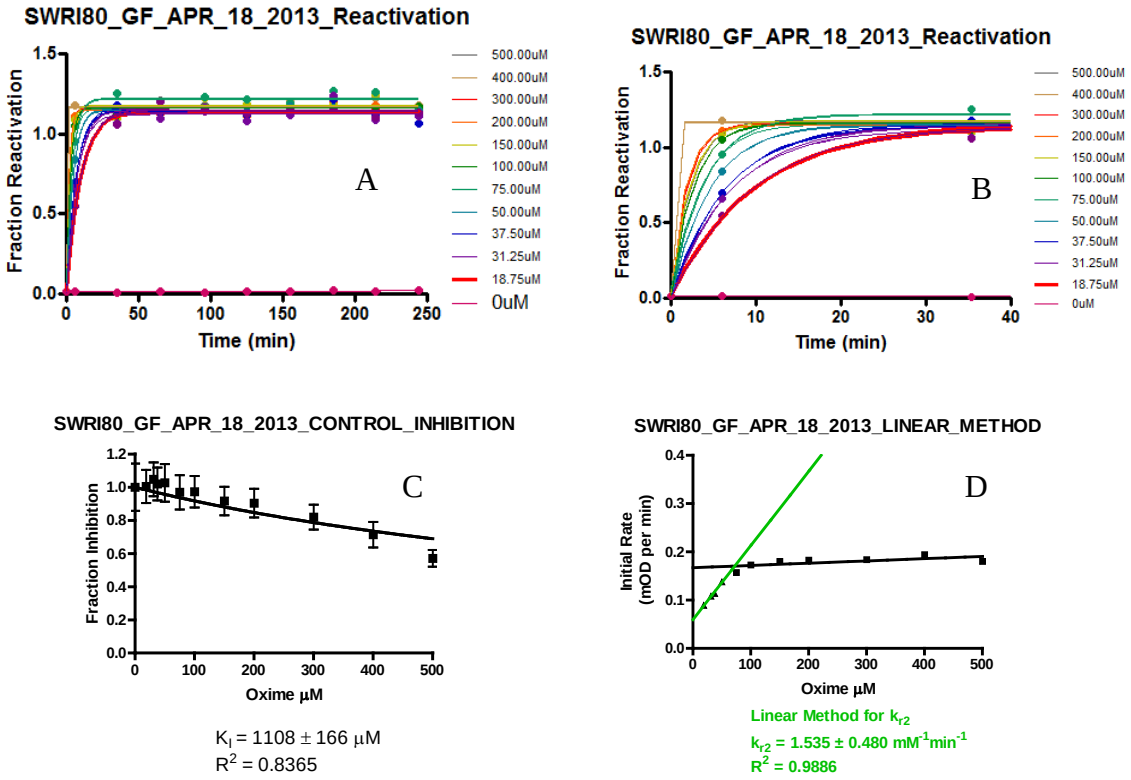


Figure 2a – Upper concentration range reactivation (A and B), Control inhibition (C) and Bimolecular Rate Constant graph (D) for 80A reactivation of GF-inhibited human AChE (Sigma Aldrich).

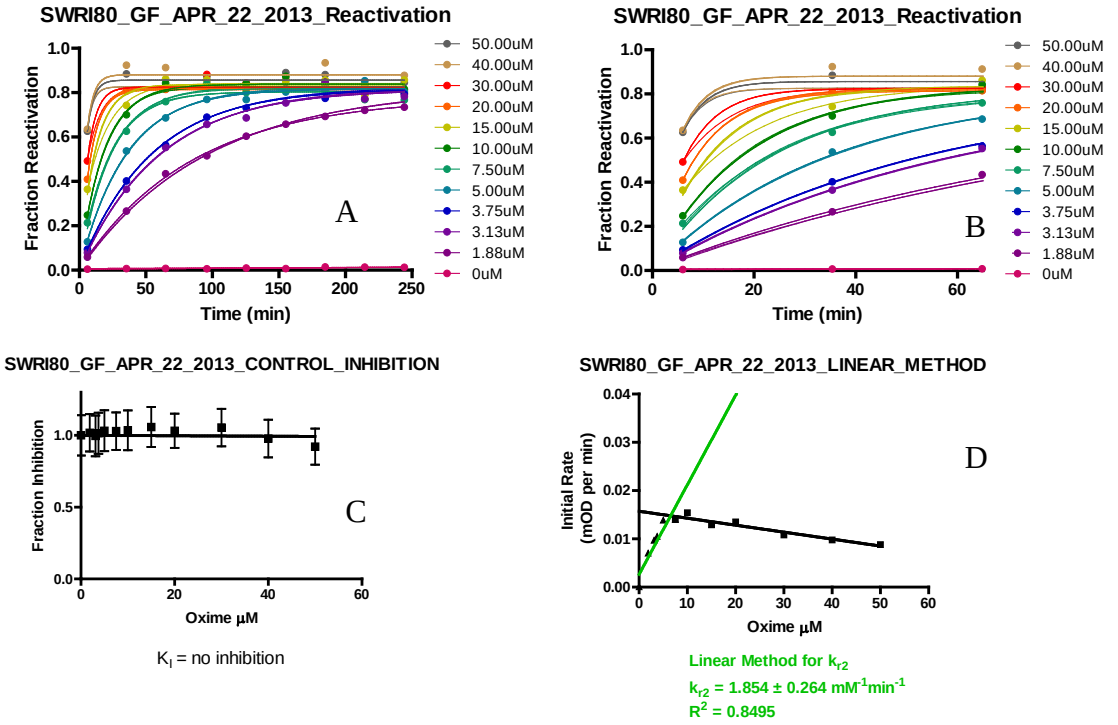


Figure 2b. Lower concentration range reactivation (A and B), Control inhibition (C) and Bimolecular Rate Constant graph (D) for 80A reactivation of GF-inhibited human AChE (Sigma Aldrich).

Reactivation of GB-inhibited enzyme by racemic 80A

Compound 80A was also tested against GB-inhibited enzyme at the lower oxime concentration range. The data are shown in figure 3. At 50 uM, although slower than GF, 80A reactivated the enzyme by 15% at 5 minutes.

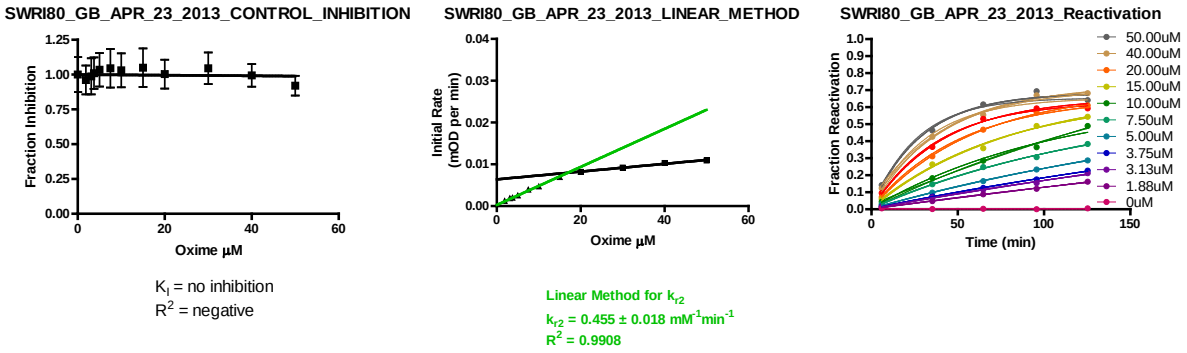


Figure 3. Control inhibition, Bimolecular Rate Constant and SwRI-80A reactivation of GB-inhibited rHuAChE. Note that 50 uM oxime gives ~15% reactivation by 5 minutes.

Initial Separation and testing of the SwRI_80A isomers

A super-critical fluid chromatography instrument was used to separate 80A dissolved in methanol and to collect peaks as they eluted. Two main peaks were well separated, but slight

shoulders (arrows in figure 4) in the peaks suggested that both the E and Z configurations were present. The shoulders were treated as parts of the main peaks (peak 1 and peak 2).

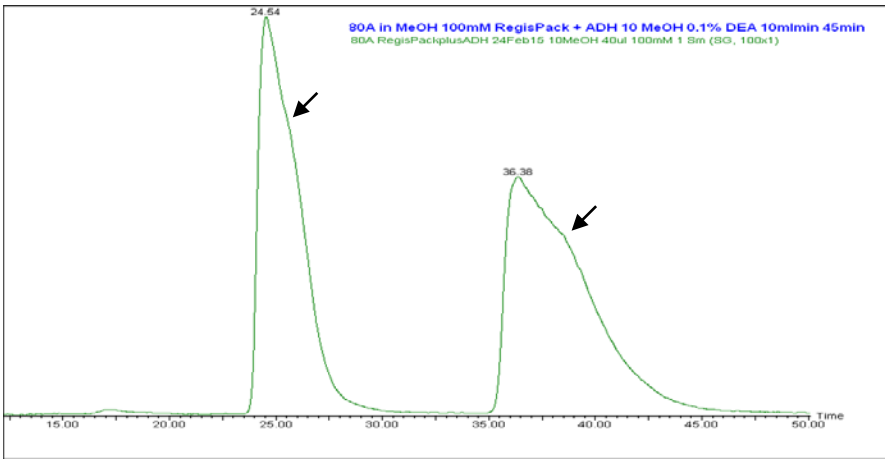


Figure 4. Two peaks eluted from the SFC column. The peaks were named according to the order that they eluted from the column (p1 at 24.5 minutes and p2 at 36.38 minutes).

The collected peaks were partially dried to remove most of the methanol and re-suspended in phosphate buffer (pH 7.4). Control (uninhibited enzyme) and GF-inhibited enzyme were prepared. Ten µM aliquots of the peaks, the racemic oxime, and a (1:1) mixture of the two peaks were prepared in pH buffer. Methanol was added to ensure testing at 1.67% MeOH. Figure 5 presents reactivation of GF-inhibited enzyme for 30 minutes.

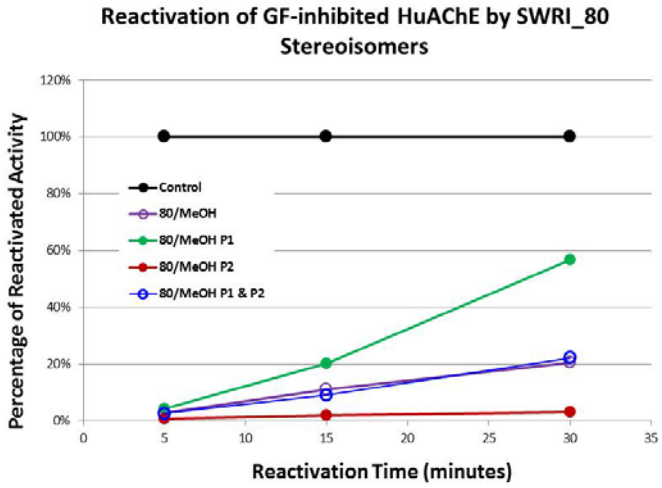


Figure 5. Reactivation of GF-inhibited rHuAChE by 5µM 80A. Note: a mixture of both isomers gave the same result as the racemic 80A. This figure shows that at least for the first 30 minutes, reactivation activity at 5 µM is due solely to peak 1.

A 2-hour reactivation test to extend the results of the 30-minute testing showed that both peaks could reactivate the enzyme, but peak 2 was a much slower reactivator (see figure 6).

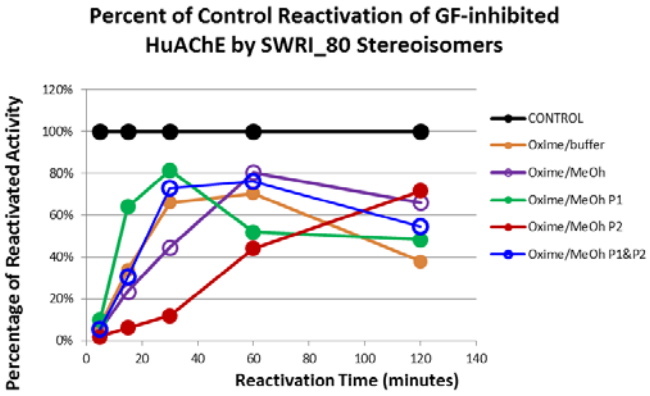


Figure 6. Reactivation of GF-inhibited rHuAChE by 5µM 80A in 1.67% methanol. Note that the mixture of both isomers gives the same result as the racemic 80A and that peak two (P2) reactivates more slowly than peak one (P1). Why the P1 reactivation drops back to about 50% at 1 hour needs to be investigated.

Testing of larger batches of the SwRI 80A isomers from two vendors

The results of the preliminary tests on a Shimadzu UV-1700 spectrophotometer provided justification for having a larger amount of 80A synthesized by SwRI. The synthesized 80A was separated by either Chiral Technologies, West Chester, PA (Batch 1), or Averica Discovery, Marlborough, MA (Batch 2). The separated batches were designated as 80A(+) and 80A(-). USAMRICD measured the optical rotations of each isomer of each batch in pure deuterated methanol. The ability of each isomer from each batch to reactivate GF-inhibited rHuAChE was measured in a side-by-side test at 50µM to compare the two batches. The results are shown in figure 7 and indicate that the batches separated by the two vendors were comparable.

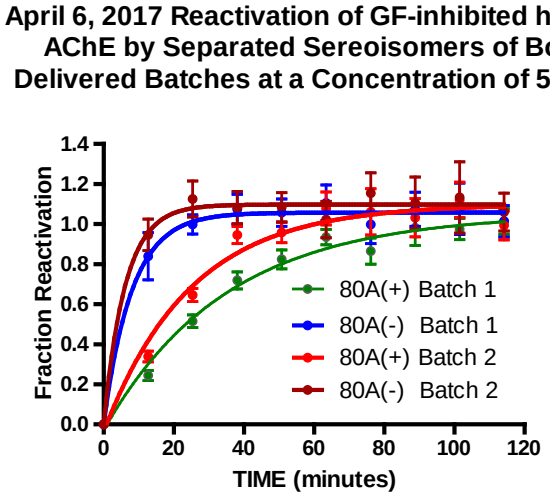


Figure 7. Reactivation of GF-inhibited rHuAChE by both batches of the separated isomers of 80A. Note that the isomer designated as (-) was the more reactive isomer in each batch. Reactivation was measured in duplicate at 50 µM on a single plate.

Kinetic testing of the SwRI 80A isomers

As is shown in figure 7, 80A(-) is the more reactive stereoisomer at 50 µM in each batch. Kinetic testing against GF-inhibited enzyme showed k_{r2} = 1.155 ± 0.278 $mM^{-1}min^{-1}$ for 80A(+) and k_{r2} = 5.08 ± 0.286 $mM^{-1}min^{-1}$ for 80A(-) . Results are shown in figures 8 and 9.

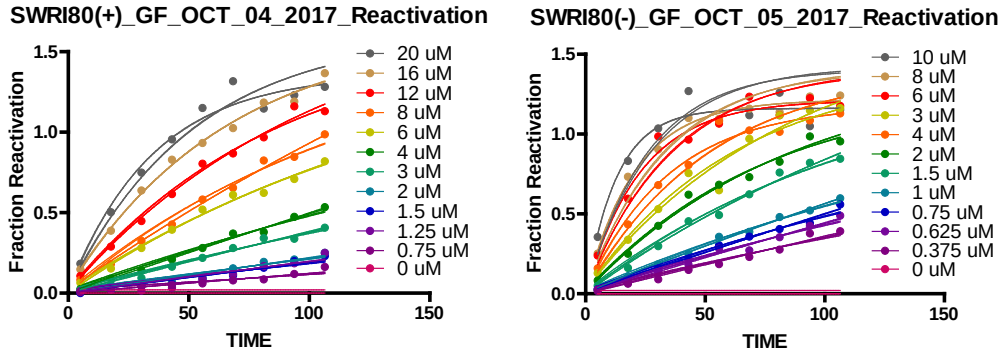


Figure 8. Reactivation of GF-inhibited rHuAChE by the isomers of 80A. Note: the (-) concentration range was lower than the (+) concentration range.

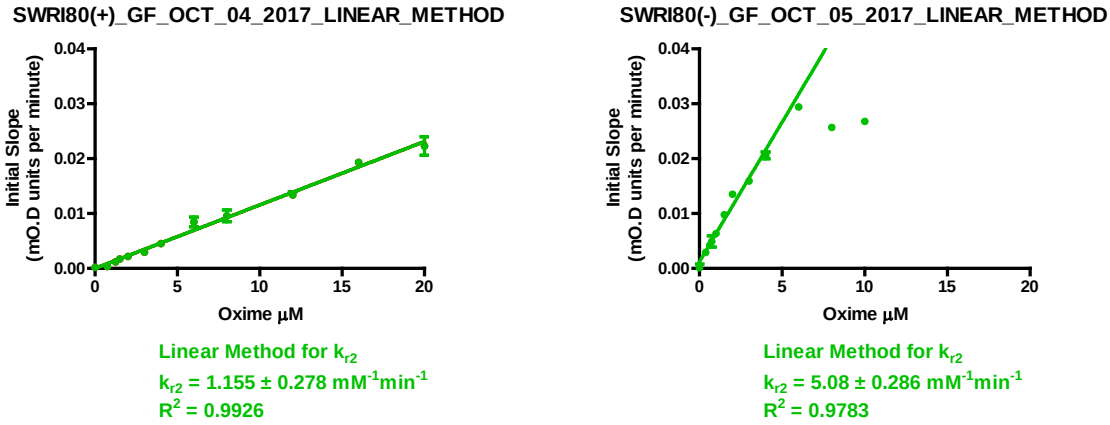


Figure 9. Kinetic results for reactivation of GF-inhibited rHuAChE by the isomers of 80A. Note: the (-) concentration range was lower than the (+) concentration range.

The numbers suggest that the 80A(-) reactivates the GF-inhibited enzyme at a rate roughly 4.4 times that of the 80A(+) isomer, but the 80A(-) rate saturates around 5 uM, while the 80A(+) rate continues to rise as oxime concentration is increased (at least up to 20 uM). The reactivation of GB-inhibited enzyme was also tested, and the results are given in figures 10 and 11.

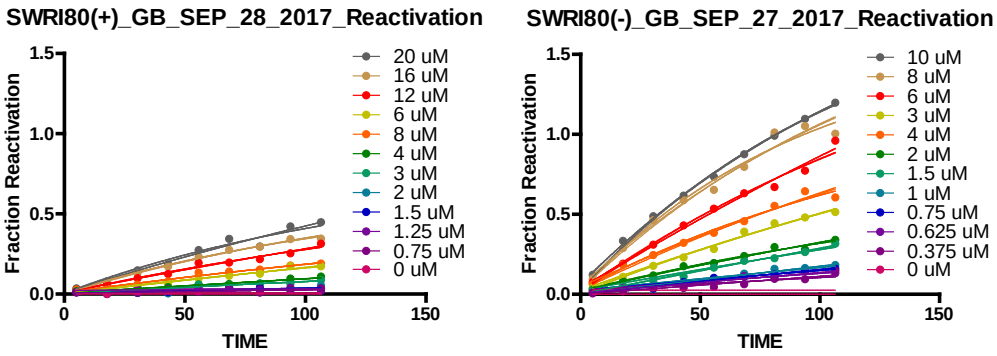


Figure 10. Reactivation of GB-inhibited rHuAChE by the isomers of 80A. Note: the (-) concentration range was lower than the (+) concentration range.

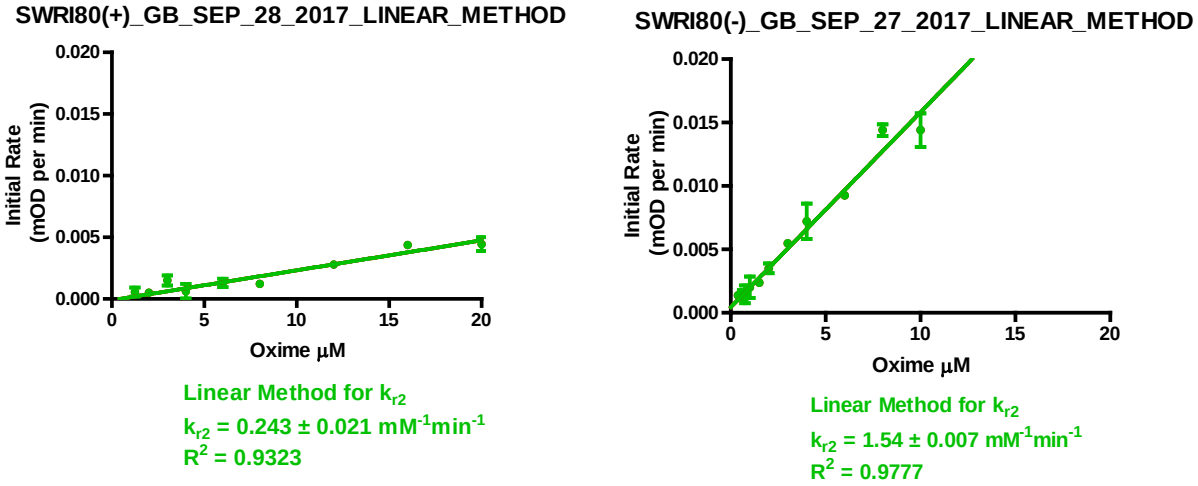


Figure 11. Kinetic results for reactivation of GB-inhibited rHuAChE by the isomers of 80A. Note: the (-) concentration range was lower than the (+) concentration range.

The numbers suggest that the 80A(-) reactivates the GB-inhibited enzyme at a rate roughly 6 times that of the 80A(+) isomer, but unlike the GF-inhibited enzyme, the 80A(-) rate is not limited at around 5 uM. The reactivation rate of GB-inhibited enzyme by 80A(+) also continues to rise as oxime concentration is increased (at least up to 20 uM).

Determining the absolute chirality the 80A stereoisomers

Without a crystal structure of an isomer of 80A, two techniques could be used to determine absolute chirality. A vibrational circular dichroism (VCD) spectrum for one isomer could be measured in the lab and compared with a theoretical VCD spectrum for either the R or S isomer that was computed using quantum theory. Computation of the VCD spectrum is computationally expensive. We chose to focus on the specific rotation of an isomer, which could also be measured in the lab and computed theoretically at much lower computational cost. Unfortunately, the theoretical results needed to be computed for each unique conformer of the molecule and used to prepare an average to estimate what was measured in the laboratory. The average was weighted by the proportion of each conformer in a solution of the isomer. There were thousands of

conformers to be considered, and the theoretical specific rotation (calculated using quantum methods) was computed for each unique one. Given the set of unique conformers for which the energy levels are known, the quantum calculations were relatively straightforward and were computed using the Gaussian16 program on the HPCMP supercomputer clusters. The determination of the unique conformers was a more daunting problem and was accomplished using tools available in the Chem3D Ultra program and by writing molecule-specific Visual Basic for Applications (VBA) code implemented in the Excel program. The final identification of absolute chirality will allow the USAMRICD to develop a chiral synthesis for the isomers and will inform docking studies for the enzyme that has been inhibited by other agents.

Structure of the SwRI_80A Reactivator

A schematic diagram of the structure of the 80A molecule is shown in figure 12. The vectors shown in the planar figure are used to define triangles in the molecule. The cross product of the two vectors that define a triangle will result in an area vector that will point directly out of the page. The angle between the area vectors for the triangle on each side of a bond was used to determine the angular rotation about that bond. When a single molecule exists in nature, the molecule is free to rotate about its rotatable bonds. Actually, all atoms are free to move, but the motions are limited by the energy that they require. For example, the atoms in the phenyl ring at the upper right portion of the figure will require a good deal of energy to move out of the plane of the ring, so the ring remains mostly planar. However the planar ring can rotate in unison about the single bond between the triangles we indicate as A_{10a} and A_{11}.

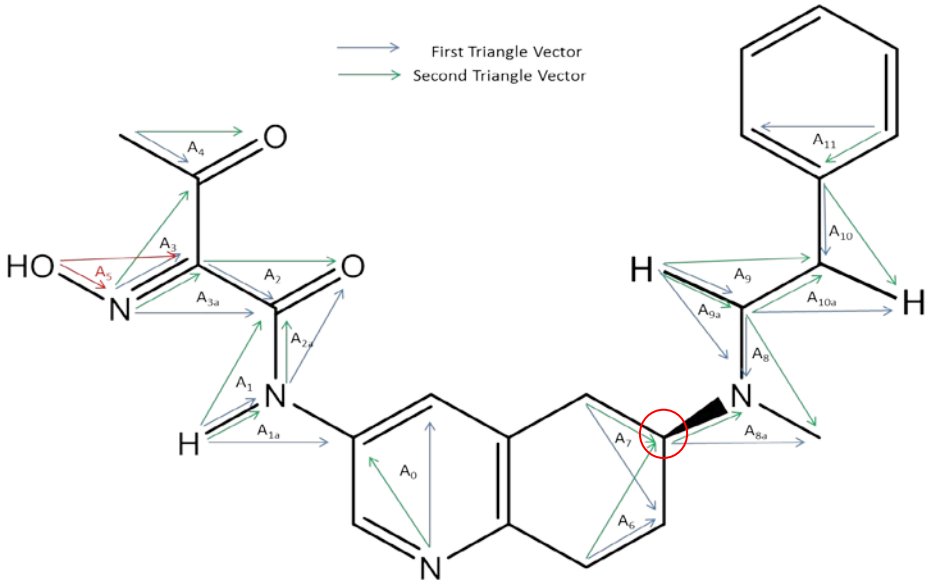


Figure 12. Structure of SwRI_80A. All unlabeled vertices are carbon atoms. The blue and green vectors are used to define two sides of selected triangular areas. We call these the triangle vectors. Each triangular area is described by a cross product of its triangle vectors. The resultant area vector points either towards or away from the viewer and is perpendicular to the plane of the figure. The angle between the area vectors at each end of a single bond defines the rotation about that bond. The atom in the hexyl ring connected to the amide nitrogen atom is a chiral center (circled in red). The vectors shown in red are used to determine the E or Z configuration of the oxime double bond.

Note that not all "H" atoms are shown in the figure (only those that participate in triangles we wish to use in describing the rotations about single bonds).

In this planar figure, all of the area vectors point directly out of (or into) the page. When the atoms in the molecule are minimized to produce a minimized conformer of the molecule where all atoms are at a minimized energy state, the area vectors will still be perpendicular to the blue and green triangles and can be used to define the angle of rotatable bonds in the molecule. For example, the angle between the area vectors marked "A_{10a}" and "A_{11}" defines the rotation about the bond from phenyl ring to the rest of the molecule. There are two isomers of the compound that exist because of the chiral center (circled in red) in the figure. The compound exists as a racemic mixture of two isomers, each with a pair of E or Z enantiomers at the C=N bond in the oxime arm. It was decided to separate the isomers of the oxime and test them individually.

Laboratory measurements of the specific rotation of each isomer

We have measured the direction in which each 80A isomer rotates polarized light. Samples of (+)80A and (-)80A were prepared at 50 mM in deuterated methanol (dMeOH). An Anton Paar (model MCP 500) polarimeter was used to measure the optical angular rotation in a 1 dm tube at 20 C for the D-line of sodium (589.3 nm). The instrument was calibrated against a quartz calibration tube. The instrument was blanked with pure dMeOH. Optical rotation readings were taken two times on different days for the samples. The readings were 1.47 ± 0.16 degrees for the (-)80A sample and -1.39 ± 0.07 for the (+)80A sample. These values were converted into specific rotations using 21.5 g/L for the concentration and 100 cm tube length. The calculated specific rotations in our lab were 68.2 ± 7.2 degrees for the (-)80A sample and -64.5 ± 3.28 for the (+)80A sample. Note that our polarimetry readings for 80A were opposite in sign to those used by SwRI to mark the supplied vials.

The energy of a molecule depends on its rotatable bonds

A molecule of 80A is free to move in solution. Conceptually, we can think of the molecule existing at any moment in a geometry defined by the instantaneous set of rotatable bond torsions for the molecule. In general, the molecule will seek a set of bond lengths, angles and rotations about its rotatable bonds where the total energy cost (interactions between its atoms and any solvent molecules) is at a minimum level. At a minimum energy configuration, small changes in any bond will cause the energy of the molecule to rise.

There are many local energy minima in which the molecule may reside. As it is lowering its energy, a molecule will end up in only one of the possible local energy minima. Each local energy minimum represents a possible stable configuration (conformation) of the molecule. It is useful to think of the molecule as "falling" down into an energy well as it continues minimization. The fall will stop at a local energy minimum. Small perturbations in atomic positions at that point will cause the total energy to rise, but unless the molecule is perturbed with enough energy to jump out of its local energy well, it will settle back into the conformation defined by that well.

A collection of molecules in solvent can be thought of as occupying many of the possible energy wells for the molecule. Note that the local energy minimum in each well may be different. It is not surprising that the lower-energy wells should have more molecules in them than the higher energy wells. The concept is illustrated in figure 13 where the abscissa is used to represent (in a

hyperspace) the geometry of the molecule. Each point on the axis represents a full set of rotations about the rotatable bonds in the molecule. The relative positions of the atoms at a single point on the axis give rise to interactions between the atoms. Those interactions contribute to the molecule's total energy at that point on the axis. Conceptually, the total energy at a specific geometry may be plotted against the ordinate at that geometry.

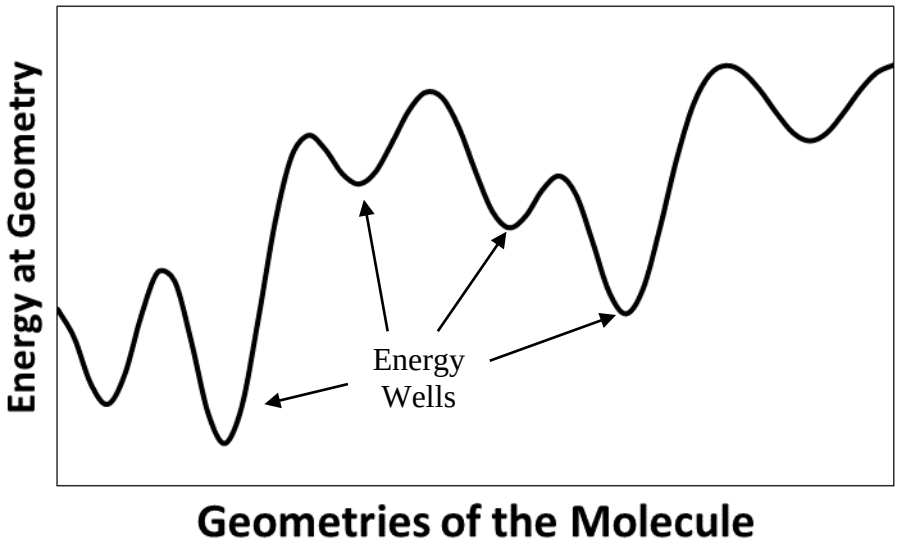


Figure 13. An illustrative energy map for a molecule showing total energy at each particular conformation.

Starting with any given set of angular rotations about the rotatable bonds in 80A, we can perform an energy minimization of the molecule. In addition to adjusting bond lengths and bond angles to the proper values, the angular rotations about the rotatable bonds will be adjusted so that the molecule will be minimized. As the molecule's geometry is energy minimized, it will "fall" into one of the possible energy wells to achieve a final energy defined by the energy at the bottom of the well. There will be as many energy wells as there are unique minimized conformations that the molecule may adopt.

Mathematical Representations of 80A using Chem3D Ultra

The 80A molecule shown in figure 12 was mathematically built using the ChemBio3D ultra (version 14) component of the ChemBioOffice Suite (CambridgeSoft, Inc.). A minimization feature allowed the molecule to be minimized by adjusting bond lengths and torsional angles about each bond and computing the total energy at each step. During the minimization, adjustments to bond lengths and torsions were made until a perturbation of any parameter caused an energy increase. The minimized energy (kcal/mole) was recorded. The "Stochastic Conformational Sampling" feature of that program was used to create 2000 minimized conformations of 80A. To generate a conformation, each atom in the molecule was displaced 100 nm in a random direction to form an "exploded" molecule. The resulting molecule was then subjected to energy minimization that continued until the energy change from step-to-step was lower than 0.01 calories per mole. Energy minimizations requiring more than 10000 steps were

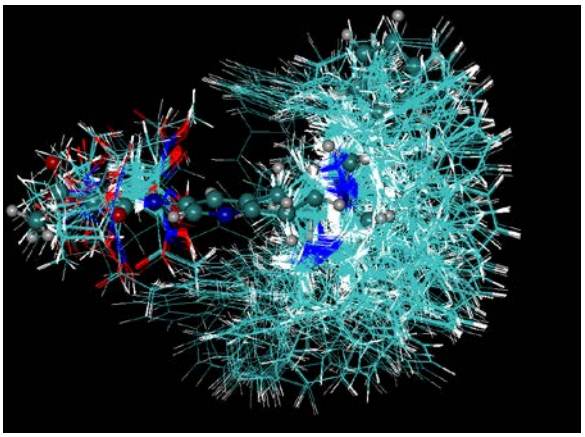
discarded. In general the minimized atomic coordinates had energy levels of between 300 and 500 kcal/mol. Occasionally, a very high minimum value was found because the molecule had minimized poorly (for example, a ring had minimized around a bond causing part of the molecule to extend through the ring). These aberrant cases were removed from further analysis. The coordinates of each minimized molecule represented a single conformation of the molecule. The final minimized energy for each conformer was stored along with the conformer.

Determination of the Unique Conformers 80A

Of course, the final minimized conformation for more than one "exploded" molecule could result in the same geometry. To account for this, the final atomic coordinates for each conformer were imported into Microsoft Excel, and a Visual Basic for Applications (VBA) macro was written to translate and rotate the conformers so that they could be compared numerically and visually (see figure 14). A second VBA macro was written and used to group the conformers into clusters that shared the same coordinates and the same minimized energy. Only a single conformer from each cluster was required. Each unique conformer was identified as either an "R" or "S" isomer by forming a plane containing the chiral atom (triangle A_7 in figure 12) and determining the position of the N atom as above or below that plane. The unique conformers were sorted by energy, and a third VBA macro was written to identify unique R conformers for which there was no corresponding S isomer of the same energy and vice versa. Lastly, a fourth VBA macro was written to create the missing isomer by changing the sign of all Z coordinates. This process resulted in 1013 "R" isomers and 1013 "S" isomers.

Visualizing the conformations

The coordinates and connections of the atoms in each conformation were output from Chem3D and imported into Excel. The listing of final energies was also imported into Excel. A VBA macro was written to rotate the conformations for better comparison. First, the coordinates of the pyridine nitrogen was subtracted from the coordinates of the other atoms, placing the nitrogen in each conformer at the origin. Each structure was then rotated about the X coordinate axis so that the carbon directly across the ring from the pyridine nitrogen was in the X-Y plane. A Z axis rotation was then applied to force that carbon onto the Y axis. Finally, the molecule was rotated about the Y axis so that the carbon in the ring connected to the amide nitrogen (a component on the A_{1a} triangle shown in figure 4) was in the X-Y plane. This procedure overlaid the conformers for viewing and a visual check of the manipulations.



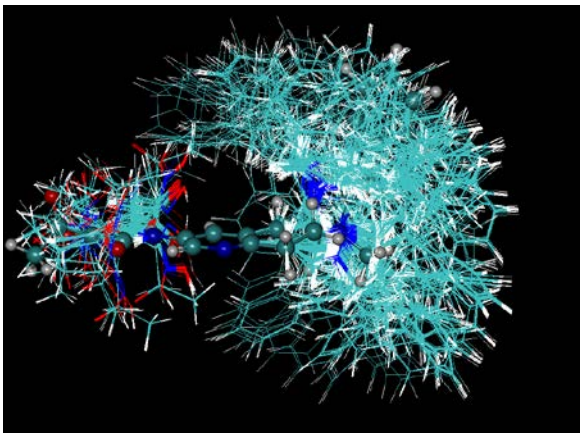


Figure 14. The overlaid conformations of the 80A R isomers (left) and S isomers (right) are shown. The amount that each conformer will rotate polarized light was determined using the Gaussian 09 software running on the HPCMP supercomputers. The lowest energy conformer for each isomer is represented in "ball and stick" mode.

Computation of the specific optical rotation for each conformer.

An Excel workbook was developed to organize the unique conformers and prepare a Gaussian 16 input job file for each one. The Gaussian input file directed Gaussian 16 to perform a minimization to determine a stationary point followed by a frequency calculation to compute the specific rotation at that stationary point. The level of theory used for these calculations was Hartree-Fock (HF) 6-311++G(3df,2pd) followed by a computation of the specific rotation at 589.3 nm at the same level of theory. Since the optical rotations that were measured at the USAMRICD were done with the compound being dissolved in methanol, that solvent was used for the theoretical computations. The set of 2026 Gaussian input files were uploaded to the DoD High Performance Computing Modernization Program (HPCMP) cluster (THUNDER at AFRL) and Gaussian jobs were started. Each computation took roughly 40 minutes of clock time to run on 1152 processors running in parallel. The total processing time was roughly 750 thousand computation hours. The Gaussian output files were downloaded back to the USAMRICD for analysis using yet another VBA macro. In the analysis, each downloaded result file was opened and scanned to find the computed specific rotation for that conformer. The specific rotations of the R isomers were paired with the energies of each R conformer and were ordered from lowest energy to highest energy. The process was repeated for the S isomers.

Contribution of a single conformer to the specific rotation for an isomer

When an amount of compound is dissolved into a solvent, the molecules will energy-minimize until they have equilibrated among the energy wells (conformations). The number of molecules in each well (conformation) will depend on the energy. More molecules will exist at a lower energy than at a higher energy. To compute the proportion of the compound at each energy level (conformation), the Boltzmann equation is used. It relates the proportion of molecules at each energy level to the difference between that energy level and the lowest energy level of all conformers (see the equations in figure 15).

The Boltzmann Distribution Controls the Population of Conformers Based on Energy

$$conformer\ 1 \leftrightarrow conformer\ 2 \leftrightarrow conformer\ 3 \leftrightarrow conformer\ N$$
$$E_1 \qquad E_2 \qquad E_3 \qquad E_N$$

$$Proportion\ of\ conformers\ of\ energy\ i = \frac{e^{-\frac{\Delta(E_i - E_{min})}{RT}}}{\sum_{j=1}^{j=N} e^{-\frac{\Delta(E_j - E_{min})}{RT}}}$$

Where E_{min} is the lowest energy of the conformers

Figure 15. The proportion of each conformer in solution is controlled by the energy (E_i) difference between the energy of the conformer and the energy of the lowest energy (E_{min}) conformer. Molecules will equilibrate among the different energy levels. The proportion in each well can be computed according to the Boltzmann formula shown where "R" is the universal gas constant, "T" is the absolute temperature and "N" is the total number of conformations.

The minimized energy of each conformation was used in the formula to predict the proportion of each conformation in a methanol solution. The Gaussian 16 program was run for each of the unique conformations to determine the specific rotation that would be imparted to polarized light passing through that conformer. Finally, the computed specific rotations for the conformations of an isomer were averaged (weighted by the proportion of the conformer in a methanol solution) into a single value that predicts the specific optical rotation of the isomer that would be measured in the laboratory. These calculation results were set up in an excel spreadsheet and ordered from lowest energy to highest energy conformers. The R isomer was predicted to have a specific rotation value of 42.11 degrees, while the S isomer was predicted to have a specific rotation value of -42.39 degrees. The contribution of each conformer as well as the cumulative contribution is show in figure 16. Note that the lowest energy conformers are on the left.

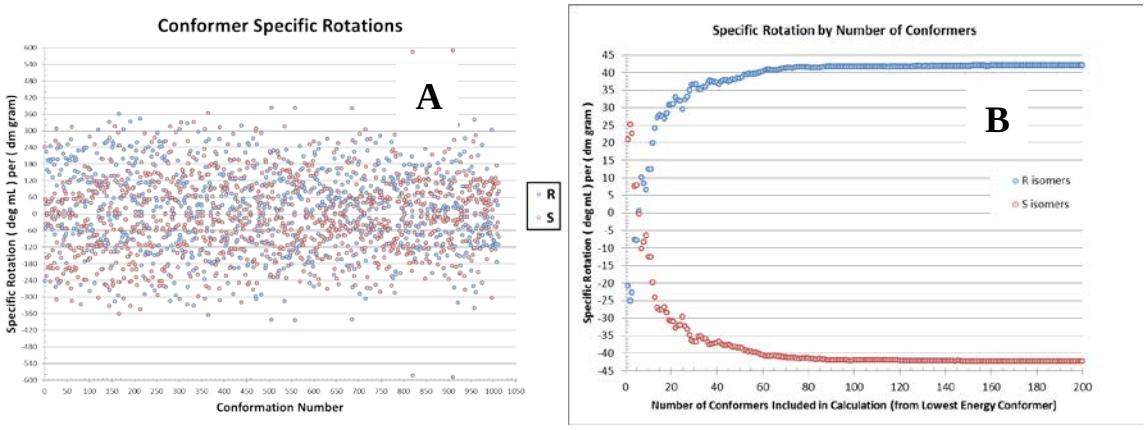


Figure 16. Panel A shows the individual conformer specific rotation by conformer for all conformers. Conformers are ordered from lowest energy to highest energy. Although the rotation for each isomer appears to fluctuate wildly, the contribution of each conformer to the total specific rotation drops with conformer energy. Panel B shows the cumulative specific rotation by conformer for the top 200 conformers. The computed specific rotation depends mostly on the lowest energy conformers. The computed specific rotation contribution to the total is negligible after about 100 conformers.

DISCUSSION

Racemic reactivations GF and GB

Racemic 80A reactivates both GF- and GB-inhibited enzyme, but reactivation of GF is considerably (4-fold) faster than the reactivation of GB. The enzyme and oxime are identical, so the difference in rates must be attributable to the difference in the groups. Perhaps an interaction between 80A and the cyclohexyl group positions the oxime for better reactivation. It is also possible that the cyclohexyl group is pulled deeper into the choline pocket than an isopropyl group, allowing 80A better visibility to the phosphorus atom (which might be blocked by the isopropyl group).

Initial separation and testing of the isomers

There was good separation of the peaks from the super-critical-fluid chromatography system at the USAMRICD (figure 4), so it is probable that each peak consisted of only a single isomer. The first peak was clearly responsible for all reactivation in the first 30 minutes, but the second peak contributed afterwards, and the contribution of the first peak was diminished. This fall-off was also seen for the racemic oxime and the 1:1 mixture of the isomers. The two batches of 80A (separated by different vendors) showed similar results. The vials marked 80A(-) were clearly faster. This indicates that peak 1 matched the vendor separated isomer marked as 80A(-). The 80A(-) isomer reactivated both GF and GB more quickly than the 80A(+) counterpart. Interestingly, the 80A(-) isomer shows saturation at a K_D around 5 μM, while the 80A(+) isomer does not. Neither isomer shows saturation against the GB-inhibited enzyme. This supports the conjecture that the cyclohexyl ring in the GF enzyme is contributing to an affinity interaction with the 80A(-) isomer, but not with the 80A(+) isomer. The interactions of the isomers of 80A docked into GB- and GF-inhibited HuAChE should be carried out as the next step in finding the answer.

Kinetic testing of the isomers

Kinetic results are given in table 1. The compound reactivated GF faster than GB, and the 80A(-) isomer was faster than the 80A(+) isomer. As might be suspected, the racemic values fall between the values for the isomers. The 80A(-) compound was found to saturate when tested against GF. This will be explored in future work with docking of the R and S isomers of 80A into molecular models of the inhibited enzymes.

		GF	GB	SCF peak matched
Racemic	80A	1.53 ± 0.480	0.455 ± 0.018	peaks 1&2 (1:1) mix
	80A(-)	5.08 ± 0.286	1.540 ± 0.007	peak 1
	80A(+)	1.15 ± 0.278	0.243 ± 0.021	peak 2

TABLE 1 - Biomolecular Rate, k_{r2} for GF and GB reactivation (mM^{-1} min^{-1})

Calculated optical rotations

The variation of the computed specific rotation depends on the geometry of the conformation and can be widely different for conformers with only a minor energy difference (ref 3, 4). We have also noted the phenomena as shown in figure 16 panel A. The widely varying specific rotations in panel A accumulate into the total contribution in panel B. The weighted contribution of the conformers drops exponentially as the energy of the conformer increases (left to right) and fewer molecules exist at that energy. After accounting for around 100 of the lowest energy conformers, the specific rotation calculation changes only minimally. The calculated values for the R and S isomers were +42 degrees and -42 degrees respectively. These values would be different if a different level of quantum theory was used. In a study comparing levels of theory, a 10- to 20-degree difference from experimental measurements was seen depending on the level of theory used, ref 4 although the sign of the prediction is correct. For computational savings, we used a relatively inexpensive theory (HF) because we are interested mainly in the sign of the predictions. We have measured 68 ± 7 and -64 ± 3 for the vials labeled 80A(-) and 80A(+) respectively. Note that our measurements were opposite to the symbol used to label the bottles. Our readings indicate that the **vial labelled 80A(-) was the R isomer and the vial labelled 80A(+) was the S isomer**. Note that a commercial vendor (BioTools, Jupiter, Florida) has confirmed this result using the Gaussian computation and experimental measurement of the much more costly vibrational circular dichroism (VCD) spectrum for 80A. ref 5

CONCLUSIONS

The primary conclusion is that the more active isomer of SwRI 80A is the R isomer that gives a positive reading on the polarimetry instrument in our lab and which has been labelled as 80A(-) on the vials supplied by SwRI. It is now apparent that molecular dynamics simulations of the 80A isomers in both GF- and GB-inhibited human enzymes should be started. The discovery studio program (ref 6) can then be used to explore the interactions between 80A and the enzymes to inform altered designs for the reactivator.

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