4-Hydroxybenzohydrazide: A Potential Reactivator for Malathion-Inhibited Human Acetylcholinesterase

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Abstract. For years, oximes are used as antidotes for organophosphate (OP) poisoning treatments. However, due to the limitations of oxime therapy, the discovery of new group of antidotes that are effective for OP poisoning treatments is desirable. A number of chemicals have been in-silico screened for their potential as malathion-inhibited acetylcholinesterase (AChE) poisoning antidotes. This screening narrows down the selection of the compounds to be synthesized, therefore reduce the time and cost needed to produce the reactivators. YASARA, a bioinformatics tool was used to perform the docking study of malathion-inhibited human AChE and reactivator-malathion inhibited AChE complexations. Fourteen potential compounds were chosen for the estimation of their binding energies and nucleophilic attack distances with malathion inhibited AChE complexes to determine their antidote capabilities. A commercially available antidote, 2-PAM was used for the comparison. Based on their energies and nucleophilic attack distance with malathion-inhibited human AChE, 4-hydroxybenzohydrazide, could also be used as the antidotes.

1. Introduction
Organophosphate poisonings are getting a lot of attention nowadays due to many incidents of improper handling and misuse of the chemicals. Every year the mortality due to organophosphate poisonings (either from dietary, household erroneous handling, accidental or/and occupational exposure) both intentionally and unintentionally, is increasing [1]. Organophosphate poisoning incidents can also occur through use of a nerve agents as chemical weapons or pesticides. This has been followed by various other incidents such as the sarin attack in the Tokyo subway in 1995, the Iran-Iraq war (1987-1988), the
assassination of Kim Jung Nam at Malaysian airport (2017) and the war in Syria (2012-2018). The latest incident was an apparent attack of a former Russian military officer, Sergei Skripal and his daughter Yulia Skripal occurred on 4th of March 2018 in Salisbury, England, involving a chemical with organophosphate intoxication symptoms.

Efforts on improving the treatment of OP poisoning cases have led to research on synthesizing hundreds of potential antidotes. Unfortunately, most poisonings from pesticides do not have a specific antidote [2]. For example, 2-PAM is very efficient in reactivating AChE inhibited with sarin or VX, but has often been unsatisfactory in the reactivation of malathion-inhibited enzyme [3]. Additionally, some of the commercially available antidotes show toxic and hepatotoxic potential [4]. Therefore, many researchers are in the quest to find a new potential antidote with low toxicity. Okinawa Institute of Science and Technology – OIST (2014) [5] reported that in-silico simulation is one of the approaches to accelerate the process to obtain the product and reduce the research costs involved in the synthesis. Examples of computer aided researches applying in-silico simulations include, inhibition of targets involved in Alzheimer disease using β-secretase (BACE-1) [6], casein kinase 1 isoforms (CK1)[7], amyloid fibril [8], and human acetylcholinesterase [9,10,11,12,13]. The tools such as virtual screening, molecular docking, pharmacophore modelling, and/or molecular dynamics were usually used in these studies.

Acetylcholinesterase is responsible for the hydrolysis of acetylcholine (a neurotransmitter) which is located at the synaptic cleft and functions to transfer signals from the nerve to the muscle cell. The hydrolysis products of this reaction are choline and acetic acid. OPs inhibit the hydrolysis of acetylcholine from occurring thus resulting in accumulation of acetylcholine in the synaptic cleft. The accumulation of acetylcholine causes continuity of signaling and leads to neurotoxicity which resulting in muscle paralysis, seizures and finally, death. All types of conducting tissue from nerve cells to sensory fibers contain AChE. The binding site of AChE acts as a charge relay system and consists of three distinct residues which are Ser203, Glu334 and His447. There are two different pockets in the binding site domain which is lined with 14 conserved aromatic amino acids [14]. OP toxicity effects on the central nerve system through the non-reversible phosphorylation of esterase when it interacts with Ser203 in the AChE molecule.

Acetylcholine is a natural substrate to acetylcholinesterase and since OP has a similar structure to this compound, it can enter the active site of acetylcholinesterase as well. The hydroxyl group of Ser203 attacks the phosphoryl group of OP and resulting to phosphorylation. Dephosphorylation of AChE can be carried out by a highly nucleophilic atom which reactivates the AChE. Ser203 and Trp86 are the key residues in inactivation and act to guard the inhibitor on the bottle neck of the binding gorge [15].Treatment of OP-inhibited AChE with pyridinium oximes i.e. LuH6 and TMB4 released of the now-functional enzyme and formed of phosphonyl oximes [16]. The reactivation mechanism has been studied for decades yet the details of this mechanism is still unclear. The aim of this study is to determine the binding energy and specific peripheral anion subsite (PAS) residues that interact with reactivators other than oximes. The study on these compounds for the reactivators has apparently never been investigated via in-silico.

2. Materials and Method

2.1 Reactivator Selection

The following compounds were chosen for the reactivator screening: 2-pralidoxime (as a control), salicylamidoxime, 4-hydroxybenzohydrazide, 6-hydroxypicolinohydrazide, pyridine-2-carboxyhydrazide, 3-pyridinecarboxyhydrazide, 2-acetylpyridine, pyrimidine, acetic acid, salicylic acid, pisolic acid, boc-nipeptic acid and indole butyric acid. These compounds represent various functional groups such as oxime, hydrazide, pyridine, pyrimidine and carboxylic acid.
2.2. Docking Study

The protein structure (.pdb) of human acetylcholinesterase which is in the apo form (PDB id: 4EY4) was downloaded from a PDB database (www.rcsb.org) while the inhibitor and reactivators (.sdf) chosen were downloaded from PubChem (pubchem.ncbi.nlm.nih.gov). Subsequently, these compounds (the enzyme, inhibitor and reactivators) were subjected to docking analysis using Autodock [17]. The parameters were set as follows: the simulation cell was interactively placed around the active site to focus docking on the most important region; certain internal degrees of freedom of the ligand to perform anything from rigid to flexible docking were fixed; and the assignment of pH dependent bond orders and hydrogen atoms were automatic. In order to determine the core fragment and its flexible attachments, automatic ligand structure analysis was used. Selected active-site residues were kept flexible during docking.

Two steps of docking were carried out; docking of inhibitor (set as ligand) with AChE (set as receptor), followed by docking of the reactivator (set as ligand) with the inhibited AChE (set as receptor). Their binding energies and nucleophilic attack distances were estimated and compared. The binding energy was obtained by calculating the energy at infinite distance between the selected object and the rest of the simulation system (the unbound state) and subtracting the energy of the simulation system (the bound state) [18]. The reactivators which have higher binding energy with low distance of the nucleophilic attack towards the inhibited AChE were selected as potential antidotes. As in some other programs, for example AutoDock, a more negative energy indicates a better binding. The reason is that these programs do not report ‘binding energies’ but the energy required to disassemble a compound into separate components which usually positive.

2.3. Validation of Docking Procedure by Superposition of the Deposited Complex Crystal Structure with Predicted Docked Structure

To validate the results obtained from the software used, the superposition of the deposited complex crystal structure of mouse AChE inhibited by sarin (PDB id: 2Y2V) with the YASARA predicted docked human AChE inhibited by sarin was carried out. The validation of the human AChE in the complex with 2-PAM (PDB id: 5HFA) with the predicted docked human AChE with 2-PAM was also carried out. The deposited crystal structures of complex AChE were trimmed. The unnecessary ligands, water, chain B and salts were deleted. Energy minimization was performed using the YASARA force field. The superposition was carried out using MUSTANG[19]. The output of the superposition value is given in random mean square deviation (RMSD). Sarin is used for the validation purpose since there is no available crystal structure has been reported on malathion-inhibited AChE so far.

3. Results and Discussion

3.1 Docking Study of Malathion towards Human AChE

The formation of the malathion-inhibited AChE complex is possible when a malathion molecule (Figure 1) enters into the active sites gorge of the AChE. It is believed that the aromatic hydrophobic residues lining along the gorge as shown by Asp74 (Figure 2), helps to steer the malathion molecule into the active sites in a reactive orientation [20]. The phosphoryl group from malathion is subjected to nucleophilic attack by the oxygen atom in the serine of the catalytic triad. As the partial bond forms between the oxygen in serine and the phosphorus in malathion, the bond between phosphorus and diethyl-2-mercaptosuccinate becomes weaker. This molecular configuration is known as a penta-
coordinate intermediate. Once the bond between the serine and malathion is fully formed, diethyl-2-mercaptosuccinate acts as a leaving group. This malathion-serine adduct is very stable and results in completely disabling the ability of the AChE to hydrolyse acetylcholine. Based on Table 1, the binding energy of malathion towards AChE with stated contacting residues was 6.38 kcal/mol. Trp86 has the nearest position towards malathion, whereas Gly448 and Ile451 are the farthest. Malathion is

| Inhibitor | Distance O-P\(^a\) | H-bond interaction | Binding energy (kcal/mol) | Contacting receptor residues |
|-----------|------------------|--------------------|--------------------------|-----------------------------|
| Malathion | 7.417            | Gly121             | 6.38                     | Trp86, Gly120, Gly121, Gly122, Tyr124, Ser125, Gly126, Leu130, Tyr133, Glu202, Ser203, Ala204, Trp236, Phe295, Phe297, Tyr337, Phe338, His447, Gly448, Ile451 |
categorized as dimethyl conjugates (malathion, dimethoate, paraoxon-methyl, dichlorvos) which are generally resistant to reactivation by oximes such as 2-PAM and obidoxime [21]. This fact supports by the high binding energy value of malathion towards AChE.

The hydrogen bond between malathion and AChE are formed by Gly121 with distance of approximately 1.97 Å. As shown in Figure 3, Gly121 form hydrogen bonding with the oxygen from malathion molecule (shown by the yellow dotted line). Ser203, Glu334 and His447 are the active site residues for the catalytic machinery of human AChE. The nucleophilic oxygen from Ser203 attacks phosphoryl group of malathion forming a bond with a distance of 7.417 Å. Trp86 is involved in the maximum π-cation interaction on the anionic subsite of the human AChE other than Ser203. This residue is also reported to guard the inhibitor at the bottle neck on the binding gorge. The role of Trp86, Tyr124 and Ser203 are to hold malathion in its binding cavity. Intramolecular π-π interaction of Phe297 is observed, whereby this interaction is significant in terms of most of the OP-AChE interaction [22] (Figure 4). The malathion-human AChE complex is stabilized by Trp86, Gly121, Gly122, Tyr124, Ser203 and His447 through hydrophobic-interactions (Figure 5).

Figure 3. Docking of malathion towards human AChE. Residues involved in the malathion-inhibited AChE complex are highlighted. Red colour represents malathion while yellow colour denotes AChE. Yellow dotted line represents hydrogen bonding while black line represents nucleophilic attack of Ser203.

Figure 4. Intramolecular π-π interaction of Phe297 with other AChE residues.
Figure 5. AChE residues that involve in the hydrophobic interactions with malathion.

3.2 Docking Study of Reactivators towards Malathion-Inhibited Human AChE

Factors contributing to the interactions between the reactivators with the OP-inhibited AChE complex are: binding capacity between the reactivator and OP, nucleophilic attack distance and interaction between peripheral anion subsite (PAS) residues of the enzyme and reactivators. The AChE has two important sites for the reactivation to occur, which are the PAS and the esteric site. The PAS of the enzyme that contribute to the interaction are these residues: Trp86, Tyr124, Phe297, Tyr337 and Tyr341, which are the most contributing residues in the hydrophobic interaction and for ligand stabilization in the peripheral site area [23]. Based on Table 2, the reactivation of 14 potential candidates with commercially available 2-PAM towards malathion-inhibited AChE shows that, 4-hydroxybenzohydrazide (Figure 6) exhibits high binding energy with shorter distance of the nucleophilic attack among the other candidates. The binding energy is 6.45 kcal/mol with the stated contacting residues. Asp72 has the nearest position towards 4-hydroxybenzohydrazide, whereas Trp286 and Tyr341 are the farthest. Asp74 appears at the gorge entrance which helps to steer the molecule into the active sites in the reactive orientation (Figure 7).

Figure 6. The ball and stick structure of 4-hydroxybenzohydrazide molecule. Colours represent: red, oxygen; purple, nitrogen; grey, carbon; white, hydrogen.

Figure 7. Molecular surface view of the docking of 4-hydroxybenzohydrazide towards malathion-inhibited human AChE. The gorge entrance of the binding pocket is shown by the red arrow. Colour represent: red, malathion; green, 4-hydroxybenzohydrazide; and yellow, AChE.
Figure 8 shows hydrogen bonding is formed between four residues of AChE with the reactivator with the distance between 1.81-2.09 Å. The structure and the position of the reactivators affect the reactivation potential. 4-hydroxybenzohydrazide is seen to have interaction with peripheral anion subsite (PAS) residues (Tyr124, Trp286 and Tyr341) from the AChE. These residues are important in the binding of the reactivators, thus exposing them to the anionic subsite where electrostatic interaction occurs. As the affinity between the reactivators and the P-site area increases, the affinity for the whole system also increases leading to stabilization [23]. Aromatic rings of Tyr124 flank the indole group of Trp286 and together they interact with charged groups of ligands [24]. Additionally, the indole ring of Trp286 displays a variety of interaction modes, including stacking, aromatic-aromatic and π-cation depending on the nature of the ligand. The nucleophilic attack of the hydroxyl group from 4-hydroxybenzohydrazide towards the malathion-inhibited AChE complex occurs with the distance of 10.92 Å (Figure 8). Hence the malathion adduct from the oxygen of the active site serine is removed and reactivate the AChE. Referring to Table 2, although the binding energy for indolebutyric acid is considered high, it shows longer distance of the nucleophilic attack. This conclude that although the reactivators may have interactions with the inhibited AChE, the reactivation may not possible. Intermolecular π-π interaction from Gly 122 towards 4-hydroxybenzohydrazide is also detected (Figure 9). 4-hydroxybenzohydrazide complex with malathion inhibited-human AChE is stabilized by Gly122 and His447 through hydrophobic-interactions (Figure 10).

**Figure 8.** Docking of 4-hydroxybenzohydrazide towards malathion-inhibited human AChE. PAS residues involved in the 4-hydroxybenzohydrazide-malathion inhibited AChE complex are highlighted. Red colour represents malathion, green and yellow colour denotes 4-hydroxybenzohydrazide and AChE respectively. Nucleophilic attack of OH from the ring of 4-hydroxybenzohydrazide towards phosphoryl group of malathion-AChE complex to free the AChE is also shown. The bonding distance is 10.92 Å. Hydrogen bonding between AChE residues and 4-hydroxybenzohydrazide as indicated by yellow dotted line.

**Figure 9.** Docking of 4-hydroxybenzohydrazide towards malathion-inhibited human AChE. Intermolecular π-π interaction of Gly 122 towards 4-hydroxybenzohydrazide is shown by red line.
Table 2. Docking of reactivators towards malathion-inhibited AChE compared to commercially available 2-PAM

| Reactivators              | Distance O-P (Å) | H-bond interactions | Binding energy (kcal/mol) | Peripheral interactions | site       |
|---------------------------|------------------|----------------------|---------------------------|-------------------------|------------|
| 4HBH                      | 10.92            | Tyr124, Arg 296      | 6.45                      | Tyr72, Asp74, Tyr124, Trp286, Tyr341 | Peripheral site |
| 2AP                       | 11.06            | Tyr124, Arg 296      | 5.72                      | Tyr124, Trp286, Tyr341  | Peripheral site |
| Salicylamidoxime          | 24.02            | Tyr124, Arg 296      | 5.32                      | Tyr124, Trp286, Tyr341  | Peripheral site |
| 2-PAM                     | 10.78            | Tyr124, Arg 296      | 5.63                      | Tyr72, Asp74, Tyr124, Trp286, Tyr341 | Peripheral site |
| Salicylic acid            | 10.63            | Tyr124, Arg 296      | 5.64                      | Tyr124, Trp286, Tyr341  | Peripheral site |
| 6-hydroxybenzohydrazide   | 10.81            | Tyr124, Arg 296      | 5.73                      | Tyr72, Asp74, Tyr124, Trp286, Tyr341 | Peripheral site |
| Acetic acid               | 22.22            | -                    | 4.65                      | -                       | -          |
| (R)Boc-nipeptic acid      | 22.21            | Tyr 124, Arg 296     | 5.38                      | Asp74, Tyr124, Trp286, Tyr341 | Peripheral site |
| (S)Boc-nipeptic acid      | 18.20            | Tyr 124, Arg 296     | 6.07                      | Tyr124, Trp286, Tyr341  | Peripheral site |
| Nicoxamat                 | 11.49            | Tyr 124, Arg 296     | 5.11                      | Tyr124, Trp286, Tyr341  | Peripheral site |
| Indolebutyric acid        | 16.00            | Tyr 124, Arg 296     | 6.32                      | Tyr72, Asp74, Tyr124, Trp286, Tyr341 | Peripheral site |
| Picolinohydroxyl          | 11.00            | Tyr 124, Arg 296     | 5.23                      | Tyr72, Asp74, Tyr124, Trp286, Tyr341 | Peripheral site |
| Pidolic acid              | 16.50            | Tyr 124, Arg 296     | 5.91                      | Tyr124, Trp286, Tyr341  | Peripheral site |
| Pyridine2-carbohydrazide  | 13.96            | Tyr 124, Arg 296     | 5.22                      | Tyr124, Trp286, Tyr341  | Peripheral site |
| Pyrimidine                | 17.53            | Arg 296              | 3.22                      | Trp286, Tyr341          | Peripheral site |

Figure 10. AChE residues; Gly 122 and His 447 that involves in the hydrophobic interactions with 4-hydroxybenzohydrazide is shown by green line.

3.3 Validation of Docking Procedure by Superposition of the Complex Crystal Structure with Predicted Docked Structure

The superposition value of RMSD 0.218 Å is recorded by the complex crystal structure of sarin-inhibited AChE with the predicted docked structure (Figure 11). Since RMSD value under 2.0 Å is considered acceptable [25] this result validates the docking protocol used. A distance value of 3.133 Å is recorded from the P (sarin) towards O (Ser203) as compared to 1.585 Å distance value of the crystal structure. The distance is within the acceptable range for atoms involved in nucleophilic attack which is less than
As described by Zhen [26]. Meanwhile, RMSD value of the superposition of crystal structure of sarin-inhibited AChE complex with 2-PAM with the predicted docked structure is 0.581 Å (Figure 12). However, a distance value of 7.860 Å is observed from O (2-PAM) towards P (sarin) of the predicted docked structure compared to the complex crystal structure of sarin-inhibited AChE with 2-PAM which gives a distance value of 5.423 Å from O (2-PAM) towards P (sarin). Although the distance value is high, this result is comparable with previous study by Paula [27] who reported the distance value of O (2-PAM) towards P (paraoxon) is 8.14 Å. This value has been verified by MCDM [28] hybrid method: Technique for Order Preference by Similarity to Ideal Solution-Analytic Hierarchy Process (TOPSIS-AHP)[29].

4. Conclusion

This study shows that the potential reactivators can be selected based on their binding energy, bound position and the distance of the nucleophilic attack of the reactivators towards the inhibited AChE. 4-hydroxybenzohydrazide which has a good binding energy value with malathion inhibited-AChE as well as shorter nucleophilic attacks distance could be alternative antidote for malathion AChE poisonings. This computational screening method is helpful to assist in narrowing down the selection of the potential reactivator. The promising results of 4-hydroxybenzohydrazide shown in this preliminary study, can be tested experimentally.
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