IJPSR (2016), Vol. 7, Issue 9 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 02 May, 2016; received in revised form, 20 July, 2016; accepted, 08 August, 2016; published 01 September, 2016

MOLECULAR DOCKING AND SITE DIRECTED MUTAGENIC APPROACH TO INVESTIGATE THE ROLE OF trp86 OF HUMAN ACETYLCHOLINESTERASE WITH ORGANOPHOSPHATES

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Keywords:

Human Aetylcholinesterase (*h*AChE), OPs, Docking, Residues, Mutation

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ABSTRACT: Docking and site directed mutagenesis approach was used to explore mode of binding and inhibition for human acetylcholinesterase (*h*AChE) and organophosphates (OPs). More than 200 OP molecules were investigated using Glide docking module of Schrodinger suit as co-crystal structure between two are not available in Protein Data Bank. In initial screening Trp86 was found to be involved in maximum Pi-Cation interaction on anionic subsite of *h*AChE other than Ser203 (Catalytic site). With extra precision glide docking Phoxim Ethyl Phosphonate (PEP) tops among 200 OPs based on glide docking score and interacted with Trp86, Gly121 and Ser203 whereas MM-GBSA score shows less binding affinity than heptenophos and dichlorovos. Trp86 preferred Pi interaction with ring bearing OPs and hydrophobic interactions with smaller OPs without ring bearing structures. Site directed mutagenesis at Trp86 (Trp86 to Ala86) shown the deterioration of the binding site in terms of size reduction, loss of electrostatic and geometric stabilization in binding cavity and significant reduction in binding of OPs in preferred orientation. Dock score of both wild and mutated *h*AChE shows a perfect qualitative agreement (R^2=64.1%) towards the study. Study suggests role of Trp86 on binding site is inevitably important for inhibition of Human AChE. This study also infers that development of antidotes could be more efficient when Tr86 is also taken into consideration during development of pharmacophores.

INTRODUCTION: Acetylcholinesterase (AChE, EC 3.1.1.7) is a serine hydrolase enzyme mainly available at neuromuscular junctions and cholinergic brain synapses. Its biological role in body is to terminate cholinergic impulse transmission by hydrolyzing the neurotransmitter acetylcholine to acetate and choline [1].

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.7(9).3802-09

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.7 (9).3802-09

AChE is very catalytic in nature [2] and being coded by AChE gene on chromosome 7 at 7q22 [3]. This polypeptide has sequence of 614 amino acid length which carries signal peptide of first thirty-one amino acids. Human Acetylcholinesterase (*h*AChE) has an ellipsoidal shape with dimensions ~ 45 Å by 60 Å by 65 Å.

The enzyme is monomer and has 12 stranded central mixed β sheets surrounded by 14 α helices. The most remarkable feature of the structure is a deep and narrow gorge of ~ 20 Å long penetrating halfway into the enzyme. It widens out closer to its base which is lined with aromatic amino acids that compose various subsites [4]. The gorge is comprised of the aromatic amino acids Tyr121, Phe330, and

Phe331 in *Torpedo californica* AChE [5]. The hydrophobic patch contains the choline binding site and other hydrophobic site and the acyl pocket. Sussman's structure of AChE has 2.8 Å resolution on X ray diffraction provided valuable insights on many fronts. The active site of AChE lies at the bottom of a deep, narrow gorge, a substantial portion of which is lined by 14 conserved aromatic residues. AChE enzyme and its inhibitors is target to many X-ray crystallographic and molecular modeling studies. The X-ray crystal structures of AChE from different species have been documented in the protein data bank e.g. 1EVE [6].

Since the nature of cholinergic neurotransmission in the animals is ubiquitous, AChE is the target of numerous pesticides, including organophosphate and carbamate insecticides. Organophosphates (OP) are esters or thiols derived from phosphoric, phosphonic, phosphinic or phosphor- amidic acid of broad class insecticides extensively used to enhance agricultural produce and household pest control. OPs have medical importance, e.g. Diisopropyl Phosphorofluoridate (DFP), Tetraethyl Pyrophosphate (TEPP), and Octomethyl Pyrophosphotetramide (OMPA) for the treatment of myasthenia gravis and an organophosphateesteret ecothiopate, still being used to treat glaucoma [7, 8]. They are also used as plasticizers, stabilizers in lubricating and hydraulic oils, flame retardants, and gasoline additives [9].

AChE inhibition leads to accumulation of acetylcholine at the synapses causing cholinergic hyper stimulation and neurotoxicity followed by loss of metabolic balance which may lead to death in absence of any effective treatment [10]. The OPs exert their main toxicological effects through non-reversible phosphorylation of esterases in the central nervous system. OPs are substrate analogues to acetylcholine and like natural substrate, enter the active site and covalently binding to serine –OH group. During acetylation, OP is split and the enzyme is phosphorylated, phosphate radicals of OP bind covalently to the active sites of the cholinesterase, transforming them into enzymatically inert proteins [11, 12]. In a study by Zheng et al., (2009) the residues Trp86, Tyr337, Phe338 and His447 are suggested as key amino acids for further study of structure-function relationships [13]. Ranjan et.al., (2015) also suggested active role of Trp86 in where this residue was actively involved in stabilizing the OP-*h*AChE complex through Pi-Cation [14].

Dephosphorylation of AChE is very slow (on the order of days), and phosphorylated enzyme cannot hydrolyze the neurotransmitter which leads to accumulation and continuous trigger the transmission of signals [15]. For this reason, it is of primary pharmaceutical interest. Its inhibition is one of the most successful strategies in the reinforcement of the cholinergic transmission.

To study binding characteristics of *h*AChE, a number of ligands have been co-crystallized with the enzyme. X-ray crystallographic structure of *h*AChE with OPs has not been documented so far and further details of binding mechanism between the two are limited. This research has focused on role of active site aromatic amino acids in inhibition and binding pattern of *h*AChE with OPs. The study included random selection of seventy five OPs commonly marketed in India, considering exposure to the population is random. Model of *h*AChE (PDB ID- 1B41) was used as reference model to execute OP-*h*AChE interaction study.

MATERIALS AND METHODS:

Preparation of protein target structure:

The *h*AChE (PDB: 1B41), target protein which was complexed with a snake venom toxin fasciculin-Ⅱ and other three ligands (resolution 2.76 Å) was retrieved from the Protein Data Bank. Structure was cleaned by removing heteroatoms, ligands and water molecules. Structure was imported to Schrodinger suit, Maestro v9.5**.** Protein Preparation Wizard [16] tool was used to optimize the structure. It comprised biological unit and assigned bond orders, zero order bonds to metals, formed disulfide bonds, , deleted water molecules beyond 5 Å from hetero groups, generated metal binding states, added missing hydrogens, completed any missing side chains and loops and Protein Preparation Wizard.

Protein Preparation Wizard has a refine function, which helped in optimization of H-bond network to fix the overlapping hydrogens. pH range was established to 7.0 and the structure was minimized by applying OPLS 2005 force field [17]. Restrained minimization was used until the average root mean

square deviation (RMSD) of the non-hydrogen atoms converged to 0.3Å.

Receptor grid preparation:

According to experimental facts active site of *h*AChE includes Ser203, Glu334, and His447 [18]- (Kryger et al. 2000. Zheng in 2009) studied active site of *h*AChE was composed of 29 residues. Based on these studies, binding site was selected for docking procedures. Residues on binding site are: Gln71-Tyr-Val-Asp-Thr-Leu76, Gly82-Thr-Glu84, Trp86- Asn-Pro88, Leu130, Tyr133, Glu202-Ser-Ala204, Trp286, Phe295, Phe297, Glu334, Tyr337-Phe338, Tyr341, Trp439, His447-Gly-Tyr449, and Ile451. Within the grid of binding site, Ser203, Glu334, and His447 amino acid residues were present and grid generation was performed using OPLS_2005.

Preparation of ligands:

Commonly used OP compounds were obtained from Pub chem database. Over 200 different variant structures were used for screening. Prior to screening, preparation of Ops structures were done using Lig Prep [19] (Schrödinger Release 2014-2: Lig Prep) module of Schrodinger suite. Ligands were imported in to Schrodinger workspace and each structure was neutralized, checked for any metal binding states, desalted, generated tautomers and 32 stereoisomers per ligand was allowed. Keeping in view of the flexibility of the rings present in each ligand and their possibility to change conformations during docking calculations, we have specified to generate1 low energy ring conformation per ligand. Finally, each ligand was energetically minimized using OPLS 2005 force field.

Virtual Screening and docking studies:

The virtual screening was performed using Virtual Screening Workflow (VSW) module of the Schrödinger Suite 2012. This workflow includes Lig Prep for ligand preparation, Qik Prop to filter out ligands based on properties, and Glide docking[21] at the three precision levels, High Throughput Virtual Screening (HTVS), Standard Precision (SP), and Extra Precision (XP). HTVS and SP modes were used for a large set of ligands and XP docking is more accurate than the above two methods. The predicted binding free energies of docked complexes were calculated by the molecular mechanics/generalized born surface area (MM/GBSA) method. After ensuring that the protein and ligands were in the correct form for docking, the previously defined binding site by Zheng (2009) receptor-grid was used which includes Ser203, Glu334, and His447 forming a catalytic triad. Glide generates conformations internally and passes these through a series of filters.

This first places the ligand center at various grid positions of a 1 Å grid and rotates it around the three Euler angles. In the first stage, basic score and geometrical filters eliminate unlikely binding approaches. Second filter involves a grid-based force field evaluation and refinement of docking solutions including torsional and rigid body movements of the ligand. The final energy estimation is done with Glide Score, and a single best pose is generated as the output for a particular ligand. Glide module of the XP visualizer analyses the specific interactions. Glide includes ligand-protein interaction energies, hydrophobic interactions, hydrogen bonds, internal energy, π-π stacking interactions and root mean square deviation (RMSD) and desolvation.

G Score = a * vd W + b *Coul + Lipo + H bond + Metal + Rot B + Site

Where, vd W => van der Waal energy;
Coul => Coulomb energy;
Lipo => lipophilic contact term;
H Bond => hydrogen-bonding term;
Metal => metal-binding term;
Rot B => penalty for freezing rotatable bonds;
Site => polar interactions at the active site; and the coefficients of vd W and Coul are: a =0.065, b = 0.130.

All the Glide docking runs were performed on i3 Processor CPU @ 2.60 GHz, with 4 GB DDRRAM. Glide was compiled and run under Linux CentOS 6.5 operating system. The output from Glide calculations were exported to .pdb format and studied for their detailed interactions at atomic level and all the images were rendered using Schrodinger's maestro interface v9.6 and Accelry's® Discovery Studio Visualizerv 3.5.0.

Inducing Alanine mutation in hACHE protein at Trp86:

Based on previous studies by Ranjan et al.2015; Kua24 et al. 2003 and Ordentlich22 et al. 1993 which suggest significant role of Trp86 in OP-AChE interaction, this study was planned in which Trp86 was replaced with a neutral amino acid Alanine (Ala86). Mutated structure was energetically minimized with OPLS 2005 force field. Another set of HTVS was executed with existing OPs ligands using virtual screening workflow (VSW) module of the Schrödinger Suite 2012. This workflow includes LigPrep for ligand preparation, QikProp to filter out ligands based on properties, and Glide docking at the three precision levels, High Throughput Virtual Screening (HTVS), Standard Precision (SP), and Extra Precision (XP).

Validation of the study:

OP ligands were screened using HTVS in two conditions: 1. Binding site having Trp at 86 position (Wildh AChE) and 2. Binding site having Ala at 86 position (mutated hAChE). Results obtained in both cases were used to do statistical calculations. Following energies were statistically analyzed: Dock score, Coul (Coulomb energy), Lipo (lipophilic contact term), H Bond (hydrogen-bonding term) and vdW (van der Waal energy). Minitab 16.0 statistical software was used to do all the calculations.

RESULTS AND DISCUSSION:

Virtual Screening and Docking:

Screening of over 200 OPs using Glide module in three different levels of docking and scoring processes were used for this study starting with HTVS, followed by Standard Precision (SP) and with Extra Precision (XP). SP based on Glide score criteria value allowed to pass only 13 molecules. The final docking with XP qualified only one molecule that is Phoxim Ethyl Phosphate.

TABLE 1: OPS BINDING WITH *h*ACHE AT STANDARD PRECISION DOCKING IN GLIDE, TOP THREE DOCKING SCORE AND MM-GBSA HAVE BEEN MENTIONED IN BOLD.

Organophosphates	MMGBSA-dG Binding	Docking score (Kcal/mol)
Phoxim ethyl phosphonate	-39.923	-6.247
Methamidophos	-33.207	-5.871
heptenophos	-53.017	-5.664
Fospirate	-34.948	-5.087
Dichlorovos	-41.297	-4.474
Pyridaphenthion	-33.207	-4.394
Dimefox	-38.427	-4.143
Cyanophos	-32.454	-3.969
Baythion	-39.288	-3.637
Coroxon	-34.732	-3.348
Vamidathion	-38.248	-3.108
Trichlorofon	-33.207	-2.301

Top three OPs based of glide docking score ranks are Phosxim Ethyl Phosphonate (-6.247 Kcal/mol), Methamediphos (-5.871 Kcal/mol) and Heptenophos (-5.664 Kcal/mol). Phoxim Ethyl Phosphate tops in ranking in Extra Precision Glide docking (-6.247 kcal/mol) however MM-GBSA binding score shows its lesser affinity (-39.923) in docked pose towards *h*AChE than that of Heptenophos (-53.017) and Dichlorovos (-41.297). Glide Docking score and MM-GBSA Binding score in recorded in **Table 1**. Docked files were observed in 3D space to study the interaction pattern. The objective during the observation was to see the involvement of Trp-86 of *h*AChE while binding with OPs. At standard precision Trp-86 had shown involvement while binding with Pyridaphenthion, leptophos, Phoxim and Phoxim Ethyl Phosphonate by Pi interactions and hydrophobic interaction of Trp86 with Trichlorofon, Vamidothion, Dichlorovos and Dimefox.

At Extra precision docking, Phoxim Ethyl Phosphate qualified among OPs and binds with wild type of *h*AChE in which Trp86 contributes the Pi-Pi interaction to benzene group of the ligand. Benzene head of the ligand immediately flips to opposite side when Trp86 is changed to Ala-86.

Partial positive charge developed on the ligand was neutralized by Tyr133. **Fig. 1** explains the binding of Phoxim Ethyl Phosphate in wild and mutated receptors model of *h*AChE. Mutation of Trp86 to Ala86 caused an electrostatic perturbation and it is noticeable that for mutation to Ala reduces the distances to the β-carbon and cannot be compared directly with key distances to the wild state of the molecule. This also lead to change in size of reside and shape of binding site which results in reduced average docking energy.

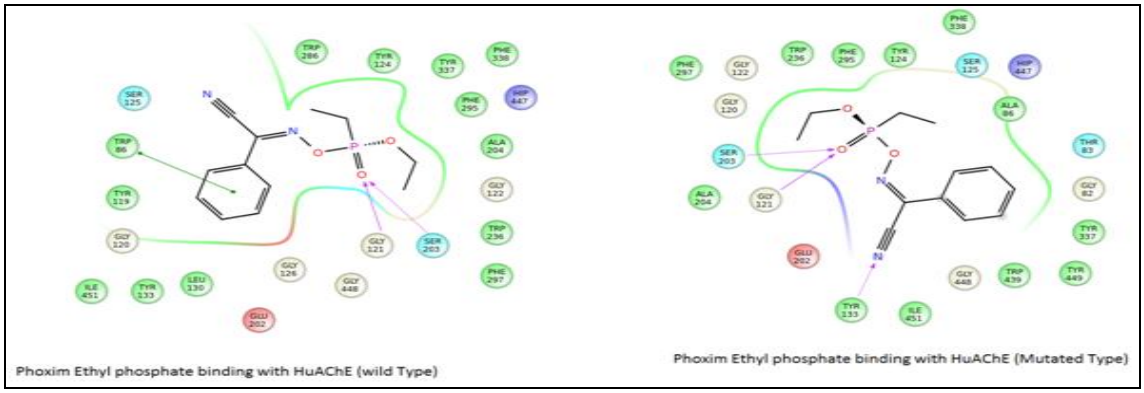

FIG. 1: PHOXIM ETHYL PHOSPHATE INTERACTING WITH *h*ACHE WILD AND MUTATED TYPE

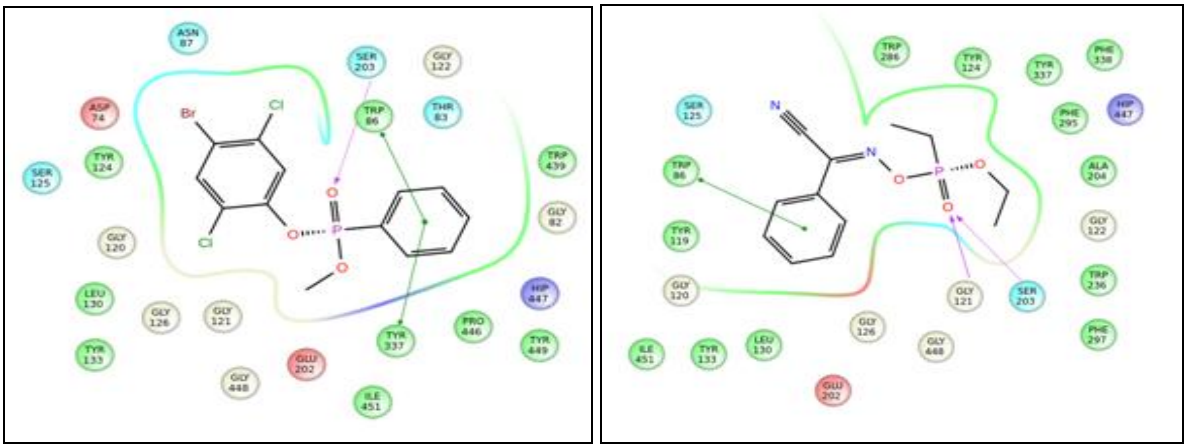

FIG. 2: LEPTOPHOS INTERACTION WITH WILD *h*ACHE (LEFT) BENZENE MOIETY OF LEPTOPHOS IS BEING INTERACTED WITH TWO AROMATIC AMINO ACIDS Trp86 and Tyr337. PHOXIM INTERACTING WITH WILD *h*ACHE (right) BENZENE MOIETY IS INTERACTING WITH Trp86

Trp86 is also involved in binding with Leptophos and Phoxim by similar mechanism (**Fig. 2**). Role of Trp86 was noticeably found in stabilizing the ring containing OPs by Pi interaction and it was also conspicuous that smaller OPs which is not bearing any ring structure with them, Trp86 interacted with them by hydrophobic interactions. **Fig.3** shows the interaction of Dichlorovos, Dimefox and Diemethoxon with *h*AChE (wild).

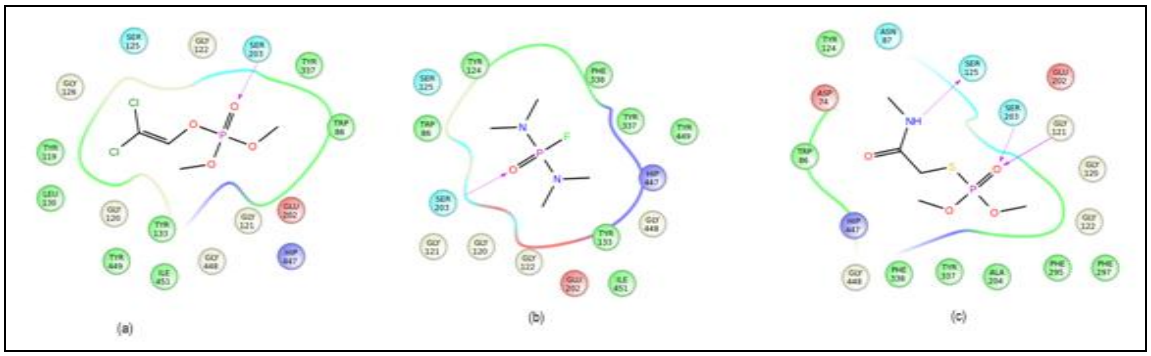

FIG.3: BINDING OF (a) DICHLOROVOS, (b) DIMEFOX AND (c) DIMETHOXON WITH HUMAN AChE.

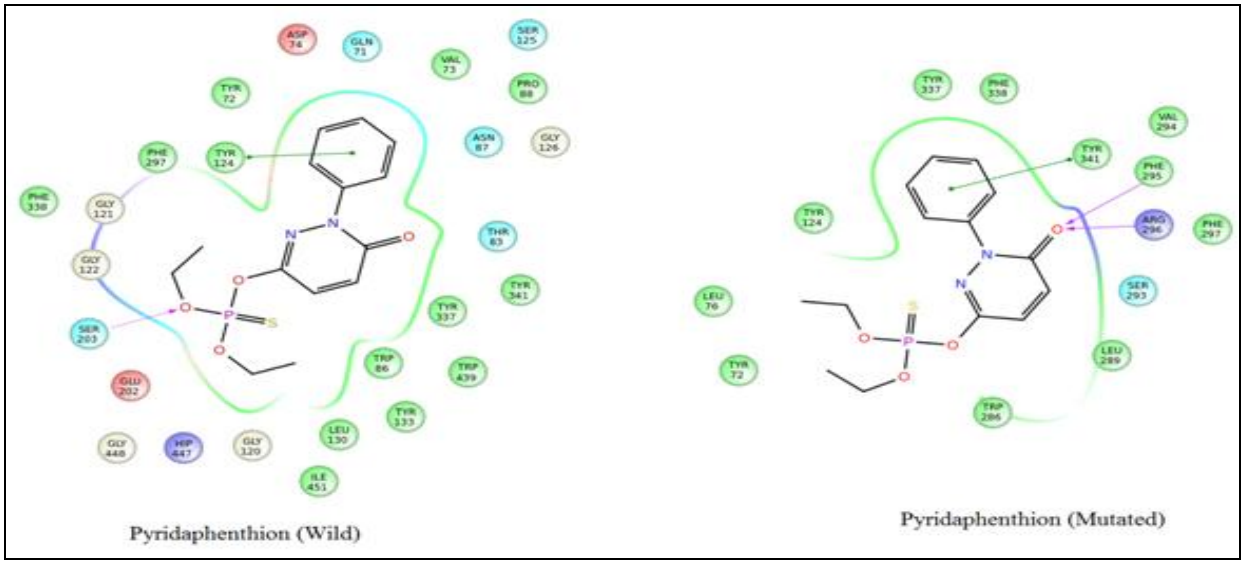

FIG.4: PYRIDAPHENTHION INTERACTING WITH *h*ACHE WILD AND MUTATED TYPE

In case of Pyridafenthion (**Fig. 4**) with wild type *h*AChE, A Pi-Pi bonding is shared by Tyr-124 to benzene moiety of this ligand and Ser-203 to oxygen of diethoxyphosphinothioyloxy group. In this binding, Trp86 is partially incorporated through hydrophobic interaction. On other hand when Trp86 is mutated to Ala86; Tyr124 which was providing Pi-Pi interaction stops its contribution and Tyr-341 takes this job which is situated exactly opposite to the Tyr-124 in 3D space. Interaction with key amino acid Ser-203 is also lost and partial positive charge generated on the molecule was normalized by Pi-cation provided by Phe-295 and Arg-296.

Correlation of Docking Energies: Energy estimation in Schrodinger was done with Glide Score which includes Dock score, Coul (Coulomb energy), Lipo (lipophilic contact term), H Bond (hydrogen-bonding term) and vdW (van der Waal energy) were used for Wild *h*AChE and mutated *h*AChE. Dock score shows healthier correlation with dock score of mutated *h*AChE with R-sq= 64.1% by equation:

Dock score Mutated = 0.5836 + 0.9333 X Dock Score Wild

Lipophilic contact terms (Lipo) shows moderate correlation in similar case with R-sq 51.7% by equation: glide_lipo (mutated) = -0.3277 +0.5494X glide_lipo (wild)

However, H bond terms shows very poor (R-sq= 8.2) and vdW (Van Der Wall energy) shows no correlation (R-sq= 0.5%) at all.

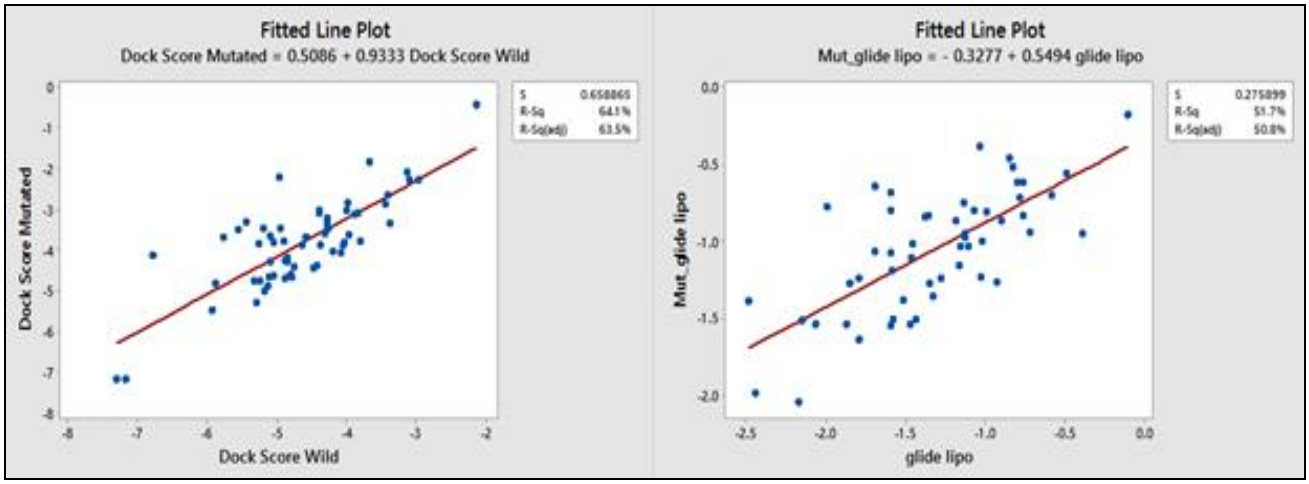

FIG.5: CORRELATION SHOWS THE EFFECT OF Trp86 ON THE BINDING SITE BY COMPARING DOCK SCORE AND LIPOPHILICITY

CONCLUSION: This study concludes that perturbation to Trp86 results in a significant reduction in binding of OPs in preferred orientation. Trp86 has imidazole ring which makes it quite larger than Alanine. The reduction in binding energy is primarily due to size of the Trp86 residue. Changing of Trp86 residue causes large reduction in size of residue. It has a combination of effect, size reduction and loss of electrostatic stabilization in binding cavity. From an energetic point of view, Trp86show the largest reduction in docking energy among the mutants. This was expected that size of the cavity would increase after mutating to smaller residues. The docking energy is reduced owing to poor stabilization by electrostatic interaction. The magnitude in docking energy reduction is small for any single mutation, because few subsidiary residues such asSer203, Phe295, Arg296 and Tyr341 on binding site also support the process. It was also noticed that the docking function used underestimated the H bond and Van Der Wall contribution. Good qualitative agreement between the obtained docking energies and lipophilicity values was found. It was also noticed in a case that charge relay center; Ser-203 stopped its contribution after mutation. OPs having aromatic rings as head show flipped head due to poor electrostatic balance.

Various reports focused on Trp86 also support the present study. Shafferman et al. (1992) in his work clearly states that replacement of Trp86 yielded inactive enzyme molecules. Kua et al., 2003 reported the importance of Trp86 along with Glu202 and Tyr337 to have proper electrostatic balance and maintained geometry of binding gorge. Ordentlich et al. 1993 reported 660 times decreased activity of enzyme after substitution of Trp86 by Alanine. Zhang et al. 2003 states Combined quantum-mechanical and molecular mechanical investigation indicate a qualitative connection between specificity (or “tightness”) of binding correlating with lowering of the reaction barrier.

Our studies on mutated model resulted in unusual binding from a geometric point of view compared with the wild. It does indicate that Trp86plays a vital role in inhibition of *h*AChE by OPs in good agreement with experimental results.

COMPETING INTERESTS: All authors declared that they have no competing interest

AUTHORS’ CONTRIBUTIONS: AR and SG carried out the molecular docking experiments and analysis of the data associated with the docking. AR,AC and AK wrote the manuscript. AR and SD co-worked on interpretation of results.TJ (supervisor) helped in guiding the process. All authors have read and approved the final manuscript.

ACKNOWLEDGEMENTS: The authors would like to thank Dr. Ashok K. Chauhan for providing the research platform. Authors also thank Amity Institute of Biotechnology for giving us access to Schrodinger suite.

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How to cite this article:
Ranjan A, Ghosh S, Chauhan A and Jindal T: Molecular docking and site directed mutagenic approach to investigate the role of trp86 of human acetylcholinesterase with organophosphates. Int J Pharm Sci Res 2016; 7(9): 3802-09. doi: 10.13040/IJPSR.0975-8232.7(9).3802-09.