

Comparative Structural Analysis of Phospholipase A2 and Combinatorial Screening of PLA2 Inhibitors

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Abstract

Phospholipases A2 (PLA2) enzyme release fatty acids from the second carbon group of glycerol. This particular phospholipase specifically recognizes the Sn-2 acyl bond of phospholipids and catalytically hydrolyzes the bond releasing arachidonic acid and lysophospholipids. PLA2 are commonly found in mammalian tissues as well as in insects and snakes venom. Venoms constitute a rich source of phospholipase A2 (PLA2) enzymes, which show remarkable diversity in their structure and function. In this investigation, we have made an attempt in analyzing the identical active domain in different PLA2 protein structure isolated from different venoms by studying the conserved active pocket residues. The 21 crystal structures of different PLA2 enzymes isolated from venoms of different species were studied and collected from PDB database. Comparative studies to analyse the conserved active site in this protein was carried out by superimposition studies using TOPMATCH server. To validate the superimposition results sequence alignment studies was carried out using T-COFFEE by multiple sequence alignment analysis. This revealed that 9 PLA2 enzymes from different venoms viz., *Daboia russellii*, *Cerrophidion godmani*, *Dienagkistrodon acutus*, *Bothrops Neuwied*, *Agkistrodon contortrix*, *Naja sagittifera*, *Bos Taurus*, *Notechis sentatusscutatus* and *Apis mellifera* showed similarity in their active pocket residues, indicating a single drug can effectively occupy their pocket and inhibit the functions of these nine proteins. Hence, in-silico drug designing studies for antivenom drugs against PLA2 was carried out by combinatorial screening of 18 antivenom compounds by docking with PLA2 molecule using Autodock 3.0 tool. In-silico drug designing studies revealed that among 18 antivenom compounds, Indole was most potent in its action in inhibiting the PLA2 function with inhibition constant of 0.04.

Keywords: Phospholipase A2, Antivenom Drugs, Superimposition Studies, Sequence Alignment, Combinatorial Screening, Molecular Docking.

1. INTRODUCTION

Phospholipases A2 (PLA2s) represents an important class of heat-stable, calcium-dependent enzymes catalyzing the hydrolysis of the 2-acyl bond of 3-n-phosphoglycerides. PLA2 enzyme releases the fatty acid from the second carbon group of glycerol. This phospholipase enzyme specifically recognizes the sn-2 acyl bond of phospholipids and catalytically hydrolyzes the bond releasing arachidonic acid and lysophospholipids. Upon downstream modification by cyclooxygenases, arachidonic acid is modified into active compounds called eicosanoids, which are categorized as inflammatory mediators.[1] The Ca²⁺ ion, an essential cofactor, and an Asp residue at position 49 are required for catalysis on artificial substrates. [2] Their catalytic activity upon cell membranes of specific tissues suggests an important role of these enzymes in venoms toxicity. PLA2 are commonly found in mammalian tissues as well as venomous insects, fish venom, frog venom and snake venom.[3] PLA2 exhibits wide varieties of pharmacological effects such as neurotoxicity, cardiotoxicity, myotoxicity, necrosis, anticoagulation, hypotensivity, hemolysis, haemorrhage and edema.[4] Envenomation due to venomous insect or snakes is a serious medical problem, especially in the farms where venomous insects and snakes are abundant. The common species such as *Crotalu rhodostoma*, *Trimeresurus albolabris*, *Daboia russeli siamensis*, *Naja atra* and other venomous insects are responsible for envenomation in Southeast Asia. [5,6] In India itself, on an average 2,50,000 envenomation are recorded in a single year. India is the richest source for poisonous species of frogs, insects and snakes. Among these majority of the bites and mortality are attributed to snake species like *Ophiophagus hannah* (King cobra), *Naja naja* (Spectacled cobra), *Daboia russelli* (viper), *Bungarus caeruleus* (Common krait), *Echis carinatus* (Saw scaled viper) etc. Among them most reports are on haemolytic venomous snakes. These snake haemolytic venoms constitute a rich content of PLA2 enzymes. Snake venom phospholipase A2 (svPLA2) can induce several additional pathophysiological effects such as cardiotoxicity, myotoxicity, pre or postsynaptic neurotoxicity, edema, hemolysis, hypotension, convulsion, platelet aggregation inhibition and

anticoagulation. [7-11] Venoms from insects, frogs, snakes, fishes constitute a rich source of PLA2 enzymes, which show remarkable functional similarity. Although, PLA2 enzyme is found in various venom derived from different organism, but possesses similarity in their substrate specificity. This reveals a hypothetical basis that this similarity is due to the conserved active site in these enzymes. In continuation with our interest to unmask the molecular basis of structural similarity and functional identity, we carried out a bioinformatics approach to study 21 PLA2 structures derived from various organisms viz., *Daboia russellii*, *Bungarus caervleus*, *Sos scrofa*, *Bothrops jararacussu*, *Naja sagittifera*, *Bos Taurus*, *Bothrops neuwiedi*, *Naja sagittifera*, *Bothrops neuweidi pauloensis*, *Vipera ammodytes meridionalis*, *Micropechis ikaheka*, *Agkistrodon contortrix*, *Echis carinatus*, *Ophiophagus hannah*, *Streptomyces violaceoruber*, *Gloydus halys*, *Dienagkistrodon acutus*, *Notechis sentatus scutatus*, *Apis mellifera*, *Cerrophidion godmani*, *Crotalus atrox* by structure superimposition studies to understand and confirm the structural relationship and analyse the conserved active site. Further, rigorous literature survey revealed that there are no reports available on the active pocket residues of PLA2 enzyme. Hence, we efficiently elucidated the active site and the residues that fall within this domain by bioinformatics approach. Combinatorial screening of 18 antivenom compounds was carried out by docking them in the elucidated active pocket by molecular docking approach to shortlist the potent compounds that can act as an effective inhibitor against all the PLA2 enzyme that possess similar active pocket.

2. MATERIALS AND METHODS

2.1. Collection of PLA2 Crystal Structures

The crystal structures of PLA2 enzyme isolated from venoms of different species were studied and collected from Protein Data Bank (PDB) database. Out of different structures present in the PDB database (www.rcsb.org/pdb), one PLA2 structure of each species present was downloaded in pdb format. PLA2 structure of *Daboia russellii* (with PDB ID - 3CBI) as a parent molecule was downloaded along with its sequence. Similarly PLA2 structures with PDB ID 2OSN (*Bungarus caervleus*), 2AZY (*Sos scrofa*), 1ZL7 (*Bothrops jararacussu*), 1TD7 (*Naja sagittifera*), 2BAX (*Bos Taurus*), 1PCQ (*Bothrops neuwiedi*), 1ZM6 (*Naja sagittifera*), 1PC9 (*Bothrops neuweidi pauloensis*), 1Q5T (*Vipera ammodytes meridionalis*), 1OZY (*Micropechis ikaheka*), 1S8G (*Agkistrodon contortrix*), 1OZ6 (*Echis carinatus*), 1GP7 (*ophiophagus hannah*), 1IT4 (*Streptomyces violaceoruber*), 1M8R (*Gloydus halys*), 1MG6 (*Dienagkistrodon acutus*), 2NOT (*Notechis sentatus scutatus*), 1POC (*Apis mellifera*), 1GOD (*Cerrophidion godmani*), 1PP2 (*Crotalus atrox*) were downloaded along with their sequence.

2.2. Structure Superimposition Studies

Structural comparison by superimposition studies was carried out by using TOPMATCH server to elucidate the conserved active site in PLA2 enzyme isolated from different organisms. [12] In this approach the PLA2 structure of *Daboia russellii* (with PDB ID - 3CBI) was used as the parent protein molecule (target) and other PLA2 structures derived from other organisms were used as query and superimposed over this parent protein molecule. The superimposed PLA2 target and query protein structures were viewed using Jmol viewer online (www.jmol.org). After the TOPMATCH was performed the superimposed structure were downloaded in PDB format and the sequence alignment were downloaded in PDF format.

2.3. Multiple Sequence Alignment Studies

PLA2 protein sequences of *D. russellii*, *B. caervleus*, *S. scrofa*, *B. jararacussu*, *N. sagittifera*, *B. taurus*, *B. neuwiedi*, *N. sagittifera*, *B. neuweidi pauloensis*, *V. ammodytes meridionalis*, *M. ikaheka*, *A. contortrix*, *E. carinatus*, *O. hannah*, *S. violaceoruber*, *G. halys*, *D. acutus*, *N. sentatus scutatus*, *A. mellifera*, *C. godmani* and *C. atrox* were collected in FASTA format from Protein Data Bank. PLA2 protein sequences similarity and availability of conserved signatures in all the 21 protein sequences was interpreted and studied by multiple sequence alignment approach using T-Coffee server (www.ebi.ac.uk/tools/msa/tcoffee) [13]. Multiple sequence alignment (MSA) file was downloaded and viewed in a color format using JalView. Results obtained by MSA study was

used to support the superimposition study and to identify active domain and the amino acid residues that fall within this domain.

2.4. PLA2 Enzyme Active Site Elucidation and Identification of Active Site Residues

The active pocket of PLA2 protein molecule was elucidated by using PDBSum of RCSB server (www.ebi.ac.uk/pdbsum/). In this approach, 17 PLA2 structures along with their inhibitors were randomly selected and studied for the amino acid residues which were interacting with the inhibitor compound as revealed by PDBSum protein-ligand interaction map. The selected inhibitor molecules varied in their interaction position. The amino acid residues that were commonly involved in interaction with all the inhibitor molecules were considered to be conserved and were representing the active site that is found to be occupied by almost all the inhibitors. This hypothesis was emphasized in elucidating the active pocket. Taking into account the number of common amino acid residues of these 17 PLA2 interacting with inhibitor ligands, the active pocket of PLA2 molecule was determined and these common interacting residues were considered as active site residues.

2.5. Study and Collection of Antivenom Drugs

Rigorous literature survey was carried out to short list potent antivenom and PLA2 inhibitors. Altogether 18 different antivenoms/PLA2 inhibitors viz., Acenaphthene, aspirin, atropine, betasitosterol, diisobutyl phthalate, diosgenin, indole, isooxazolidine, monalide, petrosaspongiolide, piperazine, quercitin, revesatrol, rosmarinic acid, rutin, tetracycline, vidadol and wedelolactone were selected and their structures were downloaded. The 3D coordinate's file of these 18 ligand molecules was generated using Prodrgr (davapc1.bioch.dundee.ac.uk/prodrgr/).[14] The 3D coordinate file compatible for Autodock 3.0 was generated and downloaded in (.pdbq) format. The 3D coordinate files of all 18 molecules were used for combinatorial screening.

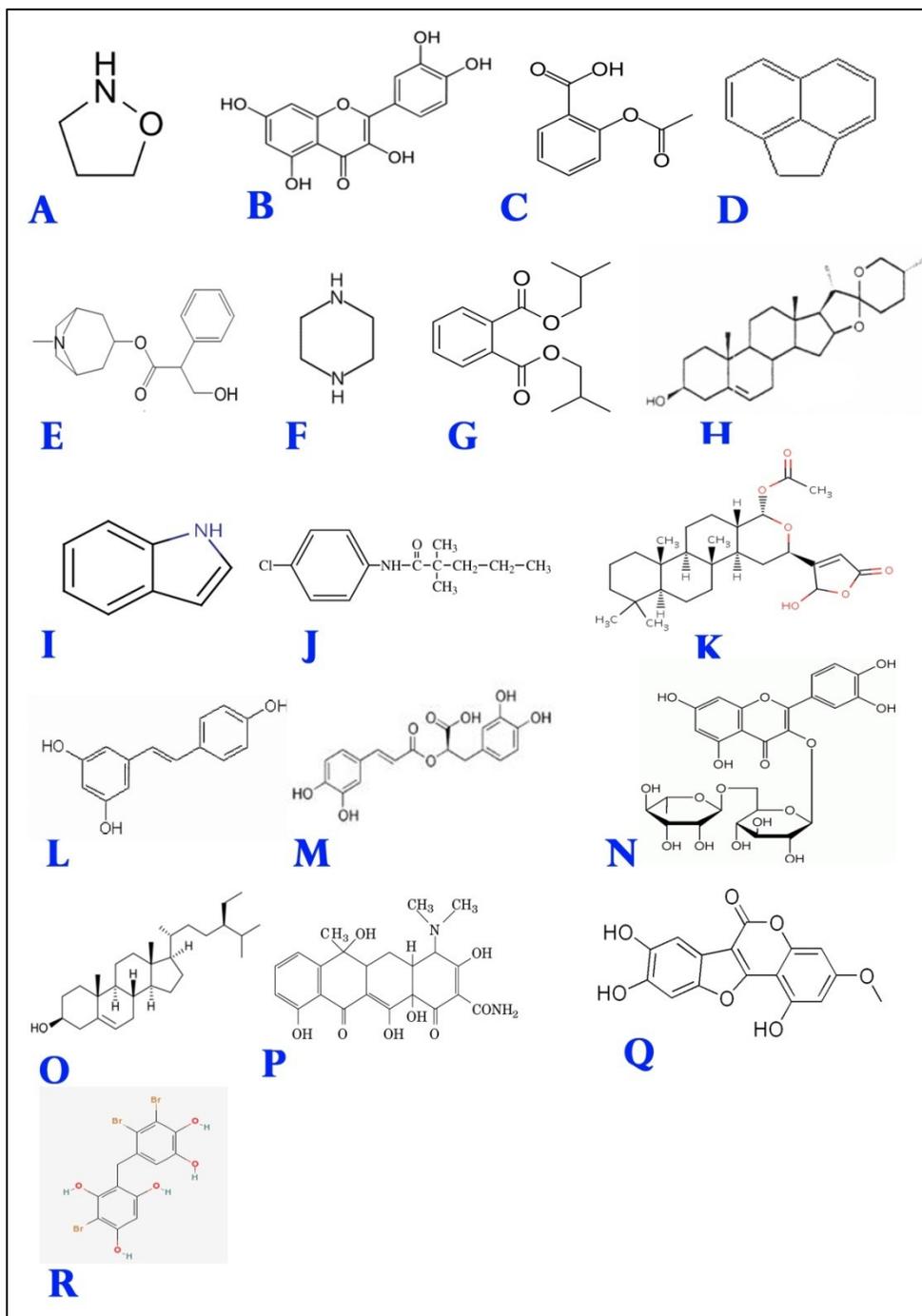


FIGURE 1: Short listed potent antivenom and PLA2 inhibitors viz., (A) Isoxazolidine, (B) Quercetin, (C) Aspirin, (D) Acenaphthalene, (E) Atropine, (F) Piperazine, (G) Di-isobutylphthalate, (H) Disogenin, (I) Indole, (J) Monalide, (K) Petrosaspongiolide, (L) Revesatrol, (M) Rosamarinic acid, (N) Rutin, (O) Betasitosterol, (P) Tetracycline, (Q) Wedelolactone, (R) Vidalol for combinatorial screening.

2.6. Combinatorial Screening by Molecular Docking Study

Automated docking was used to carry out combinatorial screening and determine the orientation of all 18 antivenom compounds bound in the active site of PLA2 protein from *Daboia russellii*. A Lamarckian genetic algorithm implemented in the AutoDock 3.0 program was employed. [15] All the 18 antivenom compounds were designed and the structure was analyzed by using ChemDraw Ultra 6.0. 3D coordinates were prepared using PRODRG server. [16] The protein structure file 3CBI was taken from PDB (www.rcsb.org/pdb), was edited by removing the heteroatoms and C terminal oxygen was added by using SPDBV tool. [17] For docking calculations, GasteigereMarsili partial charges [18] were assigned to the ligands and nonpolar hydrogen atoms were merged. All torsions were allowed to rotate during docking. The active pocket residues were predicted using CASTp server. [19] The grid map, which was centered at the following active pocket residues of the protein 3CBI [LEU2(A), GLY30(A), HIS48(A), ILE19(A), TRP31(A), ASP99(A), LYS69(A), TYR52(A), SER23(A), TYR22(A), ASP49(A), PHE5(A), ALA18(A)], and generated by applying AutoGrid. The Lamarckian genetic algorithm was applied for minimization, using default parameters. The number of docking runs was 10, the population in the genetic algorithm was 250, the number of energy evaluations was 100,000, and the maximum number of iterations 10,000.

3. RESULTS AND DISCUSSION

PLA2 is commonly found in mammalian tissues, venomous insects, fish venom, frog venom and snake venom but PLA2 from different organisms possesses similarity in their substrate specificity. Based on this knowledge, the similarity in their function and substrate specificity is due to the conserved active domain. This study was undertaken to analyze and unmask the molecular basis of structural similarity and functional identity. In the present investigation, we carried out a bioinformatics approach to study 21 PLA2 structures derived from various organisms by structure superimposition studies to understand and confirm the structural relationship and analysis the conserved active site to elucidate the active pocket residues that fall within this active domain. Further combinatorial screening of 18 antivenom compounds was carried out by docking the ligands in the elucidated active pocket by molecular docking approach.

3.1. Multiple Sequence Alignment Studies

Multiple sequence alignment by t-coffee revealed that protein with PDB ID 1T4 (violaceoruber), 1PCQ (Bothrops neuwiedi) and 1POC (Apis mellifera) did not show any relationship with the other protein sequence in the dataset. Whereas, other protein molecule showed region specific similarity. The region segment that showed maximum identity in the data set is depicted in Figure 2.

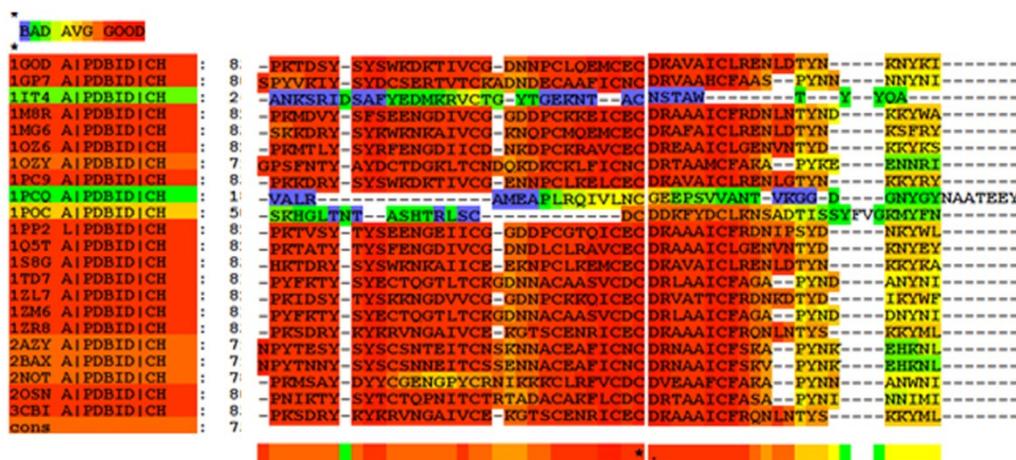


FIGURE 2: Multiple sequence alignment showing the conserved region among the 21 PLA2 protein molecules under study.

3.2. Structure Superimposition Studies

Structure superimposition study using TOPMATCH server was carried out for 21 different PLA2 structures with 3CBI of *Daboia russelli* as a target reference molecule, to which all other PLA2 structures were superimposed to identify and interpret the structurally identical region among the PLA2 enzymes. After a single run of superimposition of each PLA2 enzyme structure against the parent target PLA2 with PDB ID 3CBI, the superimposed structures were downloaded in pdf format and interpreted. Results of structure superimposition are depicted in figure 4. Further, sequence superimposition in support to structural superimposition also demonstrated similar results. The result of structural superimposition and sequence superimposition study revealed that among all the PLA2 structures used for the study, the PLA2 structures with PDB ID as 1GOD, 1MG6, 1PC9, 1S8G, 1ZM6, 2BAX, 2NOT, 1POC showed high degree of similarity in the first 122 amino acid in chain A, this is the frame within which the active pocket residue are found and hence this frame represents the active site of PLA2 (Figure 3). Further, this investigation hypothesizes the structural similarity within the active site domain reveals their functional similarity and substrate specificity. However, four PLA2 molecule with PDB ID: 2OSN, 2AZY, 1QST, 1OZ6 also showed superimposition match in last 122 amino acid sequence, but this frame is out of the active site domain.

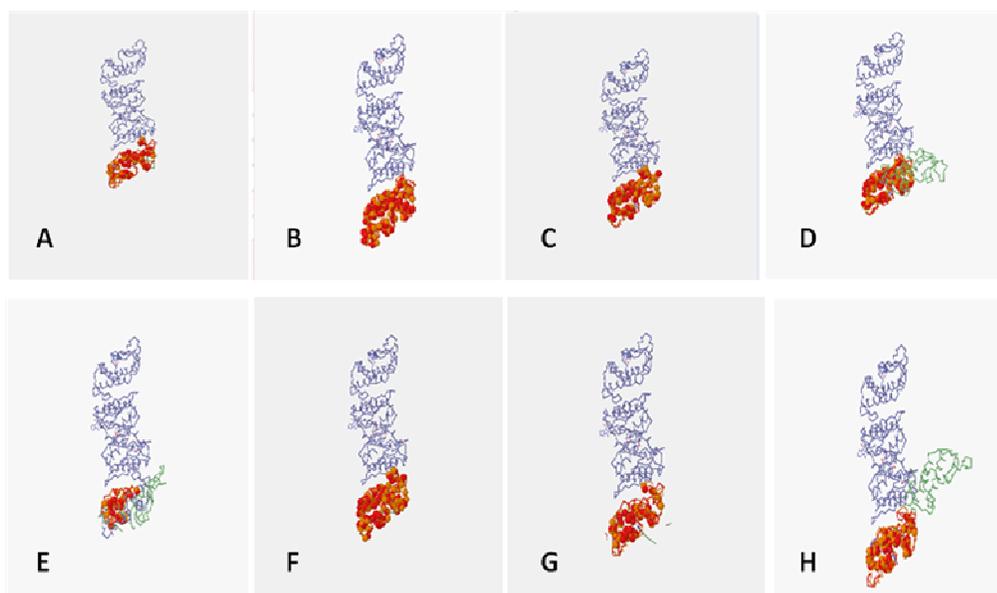


FIGURE 3: Superimposed structures showing superimposed region of 3CBI (Target) with query (A) 1GOD, (B) 1MG6, (C) 1PC9, (D) 1S8G, (E) 1ZM6, (F) 2BAX, (G) 2NOT and (H) 1POC.

This investigation support the hypothesis that, PLA2 enzyme although exists in various organism, either in venom or in mammalian tissue, their functional similarity is always documented. Through this investigation, it is presented that 8 PLA2 enzymes from organism viz., *Cerrophidion godmani*, *Dienagkistrodon acutus*, *Bothrops neuweidi pauloensis*, *Agkistrodon contortrix*, *Naja sagittifera*, *Bos Taurus*, *Notechis sentatus scutatus* and *Apis mellifera* showed active site domain structural similarity with PLA2 of *Daboia russelli*. This is in direct corroboration with the fact that active pocket's structural similarity dictates their functional similarity.

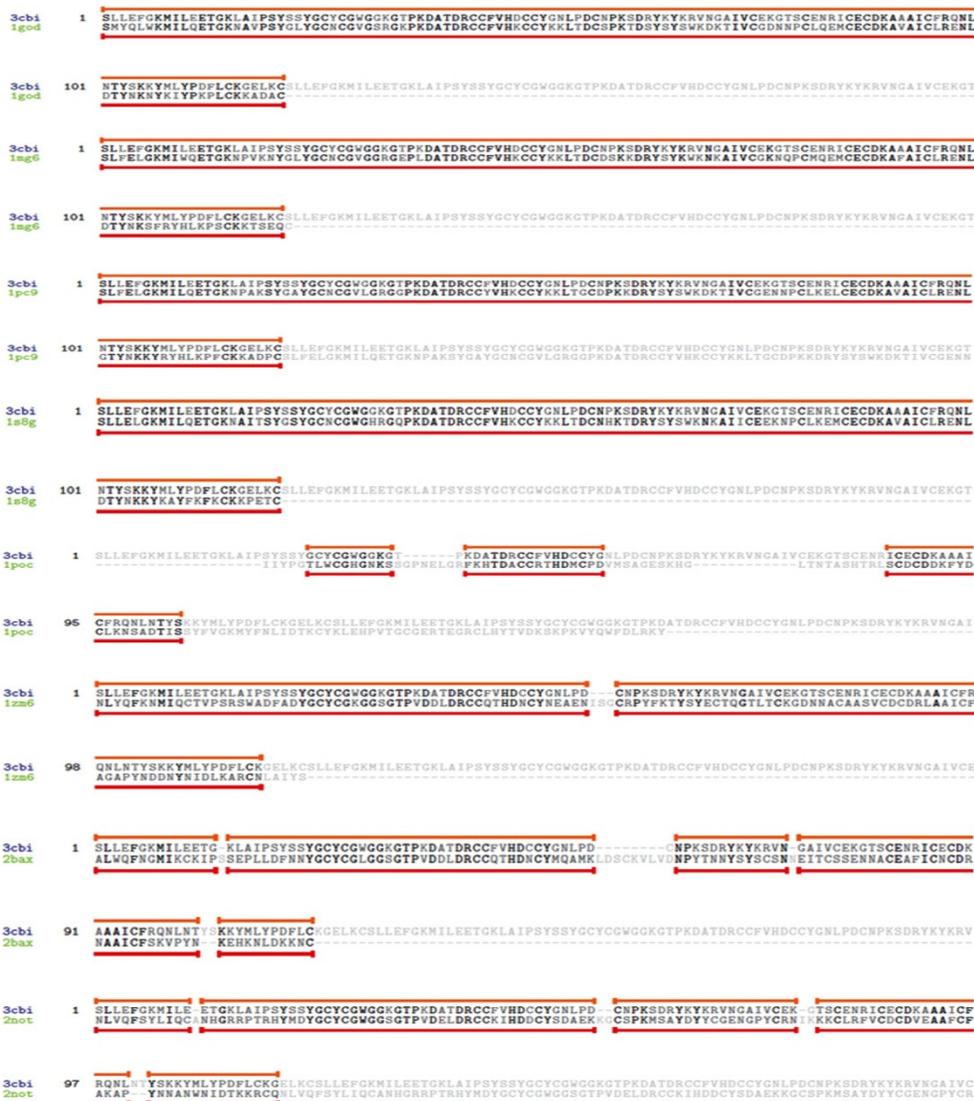


FIGURE 4: Amino acid sequence frame that falls within the superimposed region of 3CBI (Target) with query 1GOD, 1MG6, 1PC9, 1S8G, 1POC, 1ZM6, 2BAX and 2NOT.

3.3. PLA2 enzyme active site elucidation and identification of active site residues

The active pockets of PLA2 molecule of *Daboia russelli* which was used for docking study was identified using PDBSum of RCSB server. 17 different PLA2 molecule of *Daboia russelli* were randomly selected and studied for the ligand interaction. The interacting amino acid residues with different ligands, their repetition in different PLA2 structures and their respective frequency is as noted in the table. Taking in consideration the frequency of their repeat the active pocket of PLA2 of *Daboia russelli* was considered to be the domain containing following residue (Leu2(A), Phe5(A), Ala18(A), Ile19(A), Tyr22(A), Ser23(A), Gly30(A), Trp31(A), His 48(A), Asp49(A), Tyr52(A), Lys69(A), Asp99(A)).

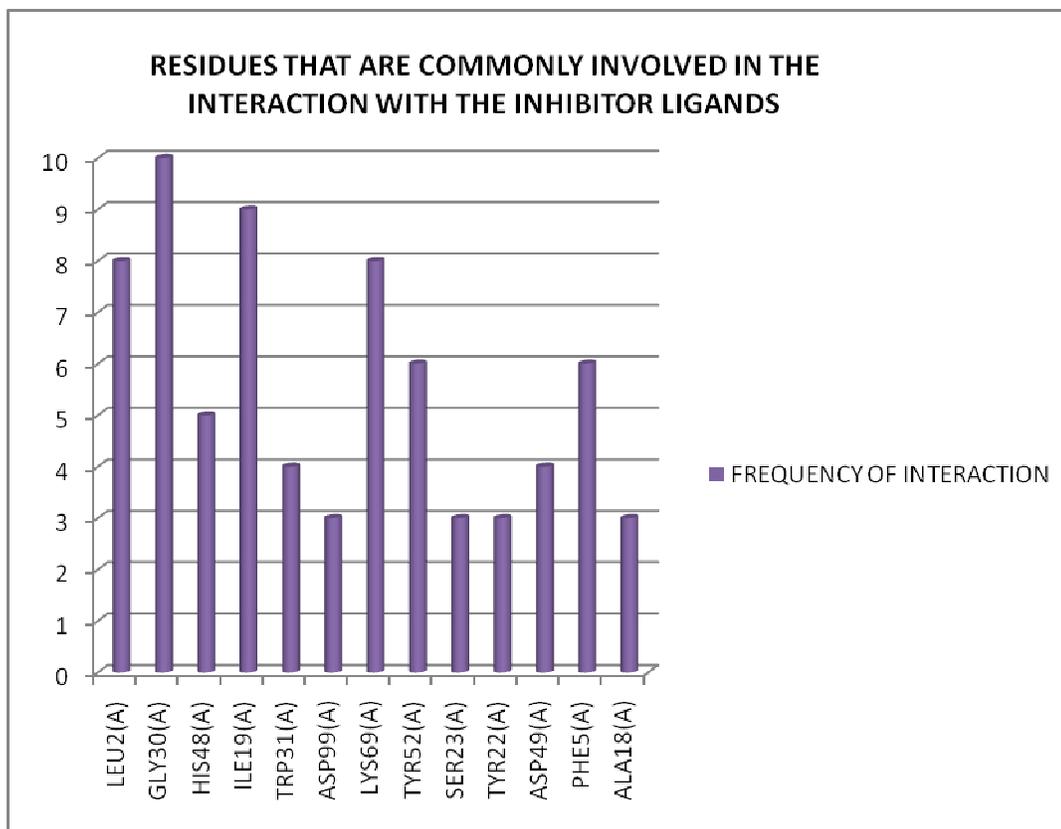


FIGURE 5: Graph showing the frequency of interaction of the residues commonly interacting with the inhibitor ligands.

3.4. Combinatorial Screening by Molecular Docking Study

Comparative computational docking of 18 potent antivenom compounds to the PLA2 revealed that Indole, Isooxazolidine, Piperazine, Diisobutyl phthalate, Tetracycline and Rosamarinic acid showed very high inhibition constant in the decreasing order (0.04, 0.01, 0.000627, 0.00546, 0.000259 and 0.000121 respectively) among all the antivenom compounds.

The docked energy for Indole was -10.55 with an estimated inhibition constant of 0.04 and intermolecular energy -2.09, while the docked energy of Isooxazolidine was -3.62 with an inhibition constant of 0.01 and intermolecular energy of -1-3.62. Indole didn't show any hydrogen bonding while Isooxazolidine showed hydrogen bonding with the backbone hydrogen of Gly 30 of PLA2.

Results of combinatorial screening revealed that among all 18 antivenom molecules Indole was found to be the most potent antivenom compound. Hence, Indole, Isooxazolidine, Piperazine, Diisobutyl phthalate, Tetracycline and Rosamarinic acid can be used to inhibit all the PLA2 enzymes from *Cerrophidion godmani*, *Dienagkistrodon acutus*, *Bothrops neuweidi pauloensis*, *Agkistrodon contortrix*, *Naja sagittifera*, *Bos Taurus*, *Notechis sentatus scutatus*, and *Apis mellifera* as they show structural similarity with PLA2 of *Daboia russelli*. Hence, they possess ligand or substrate specificity and hypothetically have the ability to bind common compound. Through this investigation it can be concluded that PLA2 enzyme from the above mentioned organisms can be inhibited using a single potent drug like Indole.

compounds	Best Orientation	Binding Energy	Docking Energy	Inhibition Constant	Intermol Energy	Torsional Energy	Internal Energy	RMS	No. of H-bonds	H-Bonding with Amino acid Residue
Piperazine	10 th	-4.37	-4.37	0.000627	-4.37	0.0	0.0	0.0	0	-
Tetracycline	4 th	-4.89	-5.55	0.000259	-5.51	0.62	0.03	0.0	4	SER23, SER23, SER23, SER23.
Rosmarinic acid	8 th	-5.34	-7.86	0.000121	-7.83	2.49	-0.03	0.0	0	-
Diisobutyl phthalate	2 nd	-4.45	-6.87	0.000546	-6.94	2.49	0.07	0.0	0	-
Indole	4 th	-2.09	-2.09	0.04	-2.09	0.0	0.0	0.0	0	-
Isooxazolidine	2 nd	-3.0	-3.62	0.01	-3.62	0.62	0.0	0.0	1	GLY30
Rutin	7 th	-5.5	-6.37	9.37e-005	-7.05	1.56	0.68	0.0	1	GLY30
Diosgenin	7 th	-9.98	-9.98	4.85e-008	-9.98	0.0	0.0	0.0	0	-
Monalide	5 th	-6.1	-6.38	3.38e-005	-7.97	1.87	1.59	0.0	0	-
Petrosaspongio lide	8 th	-8.73	-9.9	4e-007	-9.66	0.93	-0.23	0.0	0	-
Quercitin	6 th	-9.25	-9.57	1.65e-007	-9.56	0.31	-0.01	0.0	3	LEU2, TYR22, GLY30
Reveratrol	2 nd	-7.21	-8.26	5.21e-006	-8.14	0.93	-0.92	0.0	0	-
Wedelolactone	1 st	-8.39	-8.72	7.09e-007	-8.7	0.31	-0.02	0.0	0	-
Vidadol	5 th	-6.75	-7.44	1.13e-005	-7.37	0.62	-0.07	0.0	1	ASP49
Aspirin	3 rd	-5.75	-6.47	6.08e-005	-6.69	0.93	0.21	0.0	2	GLY30, HIS48
Atropine	7 th	-6.08	-7.1	3.47e-005	-7.64	1.56	0.54	0.0	2	SER23, GLY30
Betasitosterol	1 st	-7.7	-9.49	2.28e-006	-9.56	1.87	0.07	0.0	0	-
Acenaptherene	5 th	-7.48	-7.48	3.29e-006	-7.48	0.0	0.0	0.0	0	-

TABLE 1: Results of in silico molecular docking studies of PLA2 inhibitors.

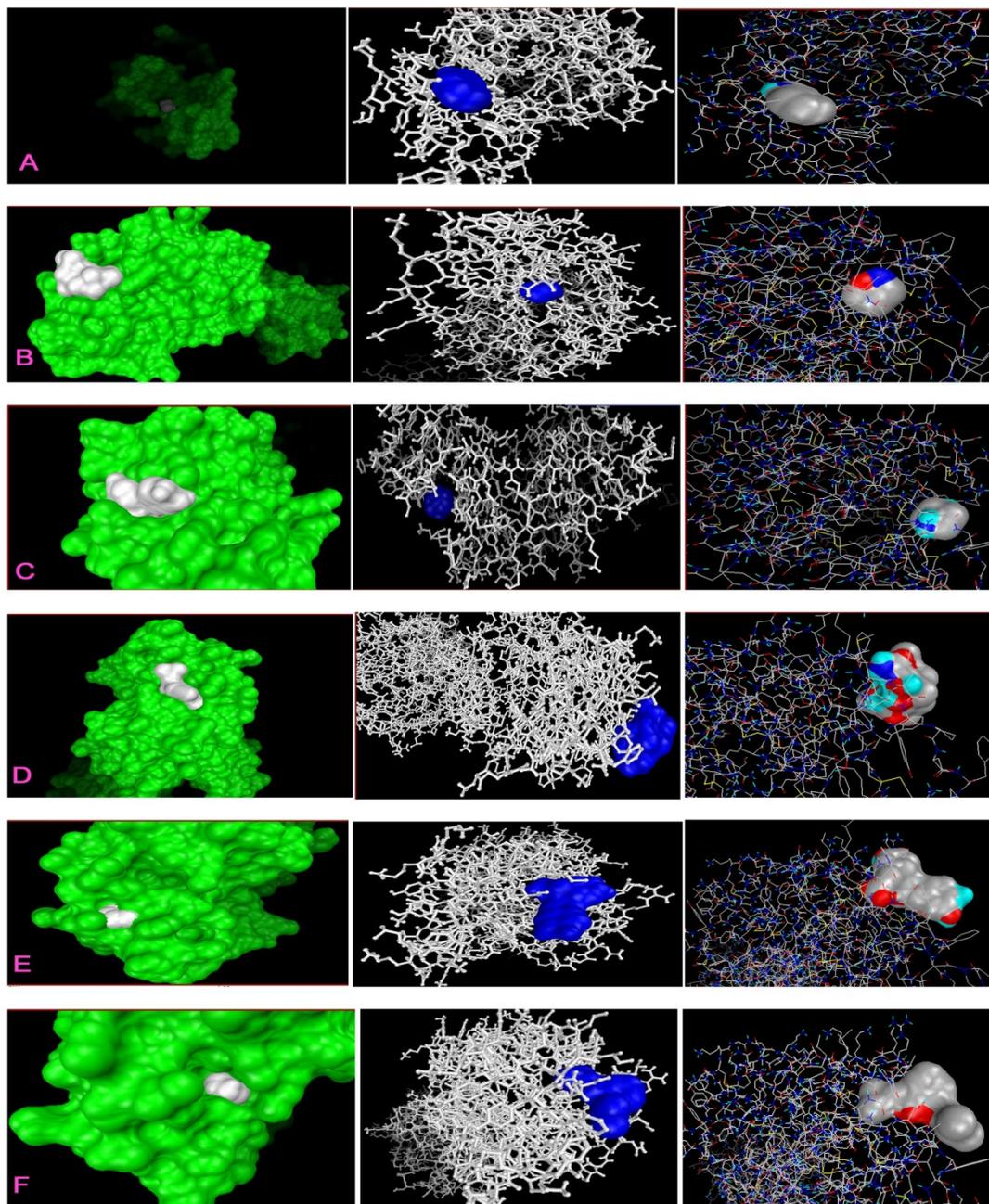


FIGURE 6: Docking of PLA2 with (A) Indole, (B) Isooxazolidine, (C) Piperazine, (D) Tetracycline, (E) Rosmarinic acid, (F) Diisobutyl phthalate.

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