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Design of new secreted phospholipase A_2 inhibitors based on docking calculations by modifying the pharmacophore segments of the FPL67047XX inhibitor

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Abstract Docking calculations that allow the estimation of the binding energy of small ligands in the GIIA $sPLA_2$ active site were used in a structure-based design protocol. Four GIIA $sPLA_2$ inhibitors co-crystallised with the enzyme, were used for examining the enzyme active site and for testing the FlexX in SYBYL 6.8 molecular docking program to reproduce the crystallographic experimental data. The FPL67047XX inhibitor was chosen as a prototype structure for applying free energy perturbation (FEP) studies. Structural modifications of the initial structure of the FPL67047XX inhibitor (IC_{50} 0.013 μM) were performed in an effort to optimise the interactions in the GIIA $sPLA_2$ active site. The structural modifications were based on: (1) the exploration of absolute configuration (i.e. comparison of the binding score of (*R*)- and (*S*)-enantiomers); (2) bioisosterism (i.e. replacement of the carboxylate group with the bioisosteric sulphonate and phosphonate groups); (3) insertion of substituents that fit better in the active site. The generated new structures exhibited higher binding energy. Such structures may spark off the interest of medicinal chemists for synthesizing potentially more active GIIA $sPLA_2$ inhibitors.

Keywords PLA_2 · GIIA $sPLA_2$ · FPL67047XX · FlexX · Molecular docking · Structure-based design protocol

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Introduction

The phospholipase A_2 (PLA_2) superfamily is a wide class of enzymes which are defined by their ability to catalyze the hydrolysis of the *sn*-2 ester bond of membrane phospholipids [1, 2]. Products of PLA_2 activity include free fatty acids, predominantly arachidonic acid and lysophospholipids. These products are further metabolized to form a variety of pro-inflammatory lipid mediators, including prostaglandins and leukotrienes, collectively called eicosanoids and platelet activating factor [2]. In particular, the arachidonic acid released from the *sn*-2 position of cell membrane phospholipids may be metabolised by 5-lipoxygenase (5-LO) to LTA_4, a substrate for the terminal enzymes of the leucotriene pathway [3, 4]; or metabolised by one of the isoforms of cyclooxygenase (COX) to PGH_2, a substrate for the terminal enzymes of prostanoid biosynthesis [5]. Lysophospholipids are metabolised to the platelet activating factor [6].

The family of mammalian PLA_2 enzymes includes three main types: cytosolic (Ca^{2+}-activated, $cPLA_2$), secreted ($sPLA_2$) and calcium-independent (Ca^{2+}-independent, $iPLA_2$) forms [1, 2, 7]. The members of the secreted PLA_2 family are small (~14 kDa) disulphide-rich proteins and consist of 10 known mammalian groups (groups IB, IIA, IIC, IID, IIE, IIF, III, V, X, XIIA). The active site of these enzymes consists of a Ca^{2+}-binding loop and a His/Asp catalytic dyad [8]. The biochemistry and biology of mammalian secreted PLA_2 have been reviewed in two recent review articles [9, 10]. Research during the last 20 years implicated the involvement of $sPLA_2$ in different inflammatory diseases including arthritis [11], asthma [12], atherosclerosis [13, 14] and sepsis [15].

The non-pancreatic, synovial group IIA PLA_2 (GIIA $sPLA_2$) cloned in 1989 [11, 16] is a low-molecular-weight

(14 kDa) enzyme with seven disulfide bonds. GIIA $sPLA_2$ is present in high levels in synovial fluid of arthritis joints [17–19]. Since GIIA $sPLA_2$ has been associated with the initiation and the propagation of inflammatory episodes, its inhibition is an attractive approach toward the development of new anti-inflammatory agents. The GIIA $sPLA_2$ inhibitors might lead to useful drugs for diseases where elevated levels of the enzyme are found. Furthermore, GIIA $sPLA_2$ are crystallized with or without ligands bound in the active site [20–22]. In addition, published research works pertain to several chemical classes of synthetic and natural PLA_2 inhibitors [23–28]. The elucidation of the PLA_2 function through the use of small molecule inhibitors is a very challenging approach. The chemical classes of inhibitors for the various subtypes of PLA_2 have been summarized in recent review articles [29–33]. Some of the most potent GIIA $sPLA_2$ inhibitors include indole scaffolds from Eli Lilly [34], D-tyrosine analogues developed by Fairlie et al. [35] and the inhibitor FPL67047XX [22]. However, optimization of the therapeutic properties of GIIA $sPLA_2$ inhibitors requires understanding of the inhibitor-enzyme interaction mechanism and establishment of effective strategies to predict the activity of new compounds. Computational chemistry approaches may help to resolve these problems.

During the last decades computational methods have experienced tremendous development. These methods are very powerful and successful in improving the understanding of structure–activity relationship of ligands and their receptors, in particular receptor-ligand complexes, and using this information to rationally modify their interactions [36–38]. Previous computational structure-based studies, focusing on the inhibitors of PLA_2 enzymes, can be divided into two groups according to the computational methods used: (1) molecular docking studies [39–41] and (2) molecular dynamics (MD) studies [42–44] which deal with modelling ligand-receptor interactions.

This paper describes the docking [45–47] calculations in order to explore the possibility of designing new FPL67047XX analogues possessing higher binding energies in the GIIA $sPLA_2$ active site. FPL67047XX can be classified as an acylamino analogue of phospholipids reported to have IC_{50} 0.013 μM against GIIA $sPLA_2$ and is co-crystallised with the enzyme [22, 48]. The strategy applied for designing new analogues consisted of the following steps: (1) the FlexX [49, 50] software in SYBYL 6.8 was tested in terms of reproducing the crystallographic data showing the binding site of co-crystallised with the enzyme ligands including FPL67047XX; (2) new designed analogues of FPL67047XX were incorporated in the active site until the achievement of better binding energy. These analogues were derived by systematic changes in structural pharmacophore segments of the FPL67047XX inhibitor.

Methods and materials

Molecular docking calculations

The crystal structures, with or without ligands bound in the active site, deposited in the RCSB protein data bank for GIIA $sPLA_2$, have been downloaded (PDB IDs: 1DB4 holo form 2.20 Å X-ray resolution [51], 1DB5 holo form 2.80 Å X-ray resolution [51], 1DCY holo form 2.70 Å X-ray resolution [51], 1J1A holo form 2.20 Å X-ray resolution [35], 1KQU holo form 2.10 Å X-ray resolution [35], 1KVO holo form 2.00 Å X-ray resolution [22], 1POE holo form 2.10 Å X-ray resolution [20], 1POD apo form 2.10 Å X-ray resolution [20], 1BBC apo form 2.20 Å X-ray resolution [21], 1N28 apo form 1.50 Å X-ray resolution [52], 1N29 apo form 2.60 Å X-ray resolution [52]). The superimposition based on C-alpha carbons of all residues showed no significant structural differences (RMSD: 0.119–0.524 Å). The 1DB4 PDB file has been chosen for the molecular docking calculations since it contains an easily manipulated single unit of the GIIA $sPLA_2$ enzyme co-crystallised with a native indole inhibitor. All hydrogen atoms were added and then assignment of the correct ionization and tautomeric states of residues such as Asp, Glu and His was achieved. All water molecules in distance greater than 6.5 Å around the ligand co-crystallised with the enzyme were deleted. The calcium ion was examined to ensure the correct atom type, the correct charge (+2) and the correct geometry (hepta-coordinated by the five oxygen atoms of residues His27, Gly29, Gly31 and Asp48 providing two metal binding sites for the ligand (see Fig. 1a).

The crystallographic ligands were extracted from the active site and the designed ligands were modelled using the SYBYL 6.8 molecular sketcher [53]. All the hydrogen atoms were added to define the correct ionization and tautomeric states, and the carboxylate, phosphonate and sulphonate groups were considered in their charged form (formal charge −1). The molecules were subjected to energy minimization using the standard Tripos molecular mechanics force field of SYBYL 6.8 molecular modelling package [53, 54] and the Powell [55] energy minimization algorithm with gradient 0.01 kcal/mol*Å was used for the minimization procedure. The low energy structures were used for the docking calculations.

The FlexX program in the SYBYL 6.8 molecular modelling package was used for the docking calculations [49, 50]. The FlexX docking algorithm allows flexibility of the ligands and keeps the receptor rigid. The default FlexX scoring function was used for the simulation. FlexX uses formal charges which were turned on during the docking run. The active site in the docking runs was set in a radius of 6.5 Å around the native indole inhibitor that existed in the GIIA $sPLA_2$ active site. All the critical amino acids

Fig. 1 **a** The active site of GIIA $sPLA_2$ where FPL67047XX inhibitor docks. The labelled amino acids and the calcium ion (hepta-coordinated by the five oxygen atoms of residues His27, Gly29, Gly31, Asp48, providing two metal binding sites, one equatorial and one axial, for the ligand binding) belong to a sphere around the inhibitor of radius 6.5 Å. **b** The binding mode of FPL67047XX inhibitor as indicated by the 2.0 Å resolution X-ray crystal structure of GIIA $sPLA_2$ co-crystallized with the inhibitor (PDB ID: 1KVO). **c** The rings' centroids and the normal vectors of the aromatic ring participating in the aromatic (π–π) stacking system. In particular, the three aromatic rings of FPL67047XX inhibitor that participate in aromatic (π–π) stacking interactions are Phenyl1, Phenyl2 and Phenyl3, and the residues Phe23 and His6

(Leu2, Phe5, His6, Ile9, Ala17, Ala18, Tyr21, Phe23, His27, Cys28, Gly29, Gly31, Cys44, Asp48, Lys62 and Phe98) including His47-Asp91 catalytic dyad and the calcium ion were included in the active site (Fig. 1a). The molecular docking process, in FlexX, is directed by the rdf-file. The rdf-file controls the protonation and contains information about the residues defining the binding site. For each residue a template has been selected that defines the properties of this residue (such as protonation, formal charges, H-bonding potential). The rdf-file also includes information about the metal ion (in this case the calcium ion). The radius "METAL_SEARCH_RAD" was used in order to determine the coordinated receptor atoms of the metal atom. FlexX takes all atoms into account in which the van der Waals radius intersects with the query radius around the centre of the metal ion. Thus the van der Waals radius of the coordinating atoms is taken into consideration (Default value: 1.0 Å).

Within the FlexX program an empirically derived scoring function is used to predict the binding energy. The physicochemical model behind FlexX can be divided into three parts: the analysis of the conformational space of the ligand, the model of protein–ligand interaction and the scoring function. The scoring function of FlexX, developed by Bohm in order to rank the solutions, is an estimation of the free-binding energy (ΔG) of the receptor-ligand complex [56].

Results and discussion

Description of the binding site of GIIA $sPLA_2$

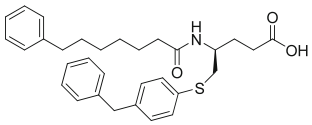
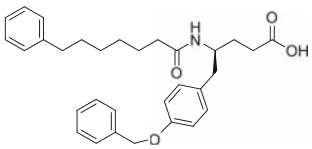
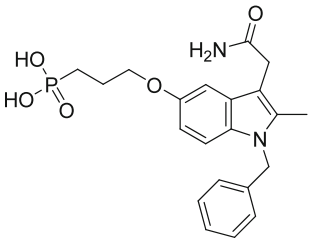
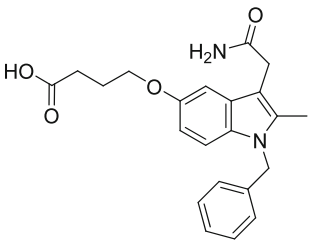
The binding site of GIIA $sPLA_2$ is composed of a hydrophobic region where the fatty acid tails of substrates bind, and a hydrophilic region which is useful for substrate cleavage. The hydrophobic region consists of aliphatic and aromatic residues within or close to the N-terminal helix (Leu2, Phe5, His6, Ile9, Ala17, Ala18, Tyr21, Phe23, Cys28, Cys44 and Phe98). The catalytic region in GIIA $sPLA_2$ is formed by the hydrophilic residues His47, Asp48, and the catalytic calcium ion which are very essential for the catalytic mechanism of GIIA $sPLA_2$. In particular, His47 and Asp48 are essential H-bond acceptors, and the calcium ion is necessary both for binding substrate and for cleavage (Fig. 1a). The calcium ion binding site is hepta-coordinated by a pentagonal bi-pyramidal cage of oxygen atoms from the residues Gly29, Gly31, His27, Asp48, and by two water molecules in the case of the uninhibited crystal structure. When the enzyme co-crystallizes with the inhibitor in the active site, the water molecules are displaced by the inhibitor [57].

Test of FlexX

The inhibitors co-crystallised with the enzyme selected to be studied for testing FlexX software were: a D-tyrosine analogue developed by Fairlie et al. (PDB ID: 1J1A), the highly potent inhibitor FPL67047XX (PDB ID: 1KVO) and two indole inhibitors (PDB IDs: 1DB4, 1DB5).

The inhibitors co-crystallised with the enzyme were docked in the GIIA $sPLA_2$ active site successfully. FlexX predicted all the main interactions and the orientation of each structure at the active site. The low RMSD (0.304–0.089 Å) values derived after superimpositions of the equivalent atoms of molecular conformation predicted by FlexX and the crystallographic conformation for each inhibitor indicate that the two conformations are almost identical (Table 1). By testing FlexX it has been proven that it is able to reproduce experimental data for GIIA $sPLA_2$ and thereafter it can be used for docking calculations in order to predict the binding energy of new analogues in the GIIA $sPLA_2$ active site.

Table 1 The RMSD values after the superimposition of the molecular conformation predicted by FlexX and the crystallographic conformation of each inhibitor

PDB ID	Molecular structure	RMSD (Å)
1KVO		0.554
1J1A		0.308
1DB4		0.190
1DB5		0.164
Mean value + SD		0.304 + 0.089

Binding mode of FPL67047XX inhibitor

Our attention has been focused on understanding the binding mode of FPL67047XX inhibitor using the 2.0 Å resolution X-ray crystal structure of GIIA $sPLA_2$ complex with the inhibitor [22]. FPL67047XX inhibitor is an extensively modified form of phospholipids and it emerges from a rational drug design effort [36] based initially on the structure of GIIA $sPLA_2$ co-crystallised with a transition state analogue (TSA) [20]. The pharmacophores that contribute to the tight binding of this inhibitor are the three phenyl rings, one carboxylate group, the amide group and one sulphur atom (Fig. 1b).

The three phenyl rings participate in extensive aromatic (π–π) stacking interactions or aromatic/aliphatic interactions with the residues Leu2 ("Phenyl3"), His6 ("Phenyl1": Rcen = 5.60 Å, $\theta = 73^{o}$) and Phe23 ("Phenyl2": Rcen = 5.44 Å, $\theta = 44^{o}$), and also participate in intramolecular aromatic (π–π) stacking interactions with each other ("Phenyl1"-"Phenyl2": Rcen = 5.22 Å, $\theta = 65^{o}$, ("Phenyl1"-"Phenyl3": Rcen = 5.07 Å, $\theta = 83^{o}$) (see Fig. 1c).

The carboxylate functionality, which exists in most of the GIIA $sPLA_2$ inhibitors, interacts with the catalytic calcium ion (O…Ca^{2+}) and participates in one hydrogen bond with Gly31 (O…H–N). The amide oxygen atom is involved in an interaction with the calcium ion (O…Ca^{2+}) and in a hydrogen bond with Gly29 (O…H–N). The amide hydrogen participates in a hydrogen bond with the imidazole nitrogen of the catalytic His47 (N–H…N) and the sulphur atom makes contact with the edge of the aromatic ring of Phe5 side chain (S…C.ar).

All the above interactions and the orientation of FPL67047XX in the active site were predicted successfully by FlexX. The distances of those key interactions, as measured by the crystal structure and as predicted by FlexX, are listed in Table 2. The differences between the crystal and FlexX distances are smaller than 1 Å, except the one between the sulphur atom and the edge of the aromatic ring of Phe5 side chain which is 1.30 Å.

Structural modifications of FPL67047XX

In an effort to optimize the enzyme-inhibitor interactions, structural modifications of the initial inhibitor have been performed. The modifications include: (1) variation of the carbon atoms between the amide group and the carboxylate group; (2) replacement of the aromatic system by aliphatic

Table 2 Measured distances of key interactions from crystallographic data versus predicted by FlexX, in the complex of FPL67047XX inhibitor with GIIA $sPLA_2$

Interaction	Crystal structure distance (Å)	FlexX distance (Å)	Difference (Å)
Ca^{2+}–COOH	2.31	2.18	0.13
Ca^{2+}–CONH	2.53	3.31	0.78
His47-NH	1.84	2.12	0.28
Gly29-CONH	2.23	1.63	0.60
Gly31-COOH	2.57	2.79	0.22
Phe5-S	4.35	5.65	1.30
His6-Phenyl1 (Rcen)	5.60	6.01	0.41
Phe23-Phenyl2 (Rcen)	5.44	5.46	0.02
Leu2-Phenyl3	3.95	3.52	0.43

chains; (3) use of (*R*)-enantiomer of FPL67047XX inhibitor; (4) decrease of the carbon chain length between the amide group and the aromatic ring R^1; (5) insertion of substituents in the para-position of the aromatic ring R^1; (6) replacement of the carboxylate group by bioisosteric groups.

Variation of the carbon atoms between the amide group and the carboxylate group

Docking calculations of the studied analogues in the active site of GIIA $sPLA_2$ have been performed. Decrease or increase of the alkyl chain between the amide group and the carboxylate led to lower binding energy confirming that three carbon atoms length is optimal for the activity. The FPL67047XX inhibitor was first tested by Baeten et al. [48]. The derivative which contained two carbon atoms between the amide group and carboxylate showed about three times less inhibitory activity. These results are confirmed by the current docking studies. In addition, we tested a derivative which contained four carbon atoms between the amide group and carboxylate. This molecule showed less binding energy indicating that FPL67047XX inhibitor has the optimal three carbon atoms distance between the amide and carboxylate group.

Replacement of the aromatic system by aliphatic chains

Replacement of the aromatic system by aliphatic chains resulted in lower binding energy for these analogues due to their inability of aromatic (π–π) stacking interactions (Fig. 2). Attractive aromatic (π–π) stacking interactions are one of the major forces governing molecular recognition. Burley and Petsko [58] have reported that aromatic (π–π) stacking interactions in proteins operate at distances (Rcen) of 4.5–7.0 Å between the centre mass of the rings, the rings' centroids. The angle (θ) between normal vectors of interacting aromatic rings is typically between 30° and 90°, producing a "tilted-T" or "edge-to-face" arrangement of interacting rings. Hunter and co-workers [59] have reported that aromatic (π–π) parallel stacking interactions ($\theta < 30^o$) between phenylalanine residues in proteins are also favourable if the rings are offset from each other. Recent ab initio calculations on benzene dimers have estimated that "tilted-T" and "off-set" parallel aromatic (π–π) stacking interactions have stabilization energies of 2.7 and 2.8 kcal/mol, respectively, suggesting that these may be very important non-covalent interactions [60].

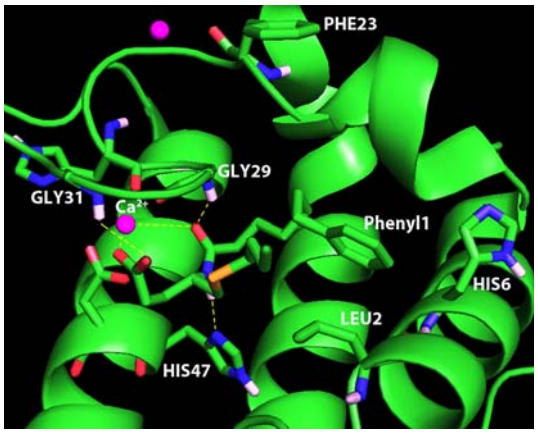
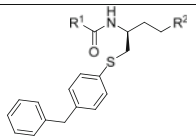


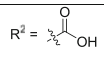
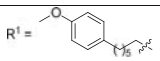
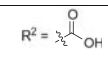
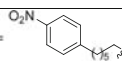
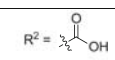
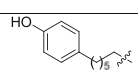
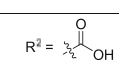
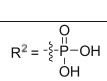
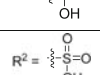
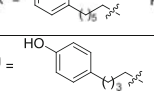
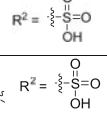
Fig. 2 The figure reveals the binding mode of a molecular structure accruing by replacement of the Phenyl2 and Phenyl3 rings (see Fig. 1c) with an aliphatic chain. The replacement is detrimental to the binding energy ($\Delta G_{FlexX} = -15{,}23$ kJ/mol) because the aliphatic chain lacks aromatic (π–π) stacking interactions

Enantioselectivity

Evidence to support the enantioselectivity of the inhibition of PLA_2 enzyme by various compounds was reported by Bennion et al. [36]. Bennion has also reported that there is at least 20-fold difference in the inhibitory activity presented by the (*R*)- and (*S*)-enantiomers of the reported $sPLA_2$ inhibitors, with the more active enantiomer possessing the (*R*)-configuration. Based on this evidence, the (*R*)-enantiomer (molecular structure **2**, see Table 3) of FPL67047XX has been tested. This shows better fitting in

Table 3 Molecular structures of FPL67047XX and its analogues and their associated binding energies



			ΔG_{FlexX} (kJ/mol)
1	R^1 = 	R^2 = 	-18.96
2	R^1 = 	R^2 = 	-19.50 (*R*)
3	R^1 = 	R^2 = 	-22.02
4	R^1 = 	R^2 = 	-22.23
5	R^1 = 	R^2 = 	-20.25
6	R^1 = 	R^2 = 	-27.61
7	R^1 = 	R^2 = 	-19.76
8	R^1 = 	R^2 = 	-22.05
9	R^1 = 	R^2 = 	-29.10 (*R*)

the active site of GIIA sPLA$_2$ by optimizing the interactions of the carboxylate group with the calcium ion (O…Ca^{2+}) and Gly31 (O…H–N) (see Table 4). The sulphur atom is at a closer distance to the edge of the aromatic ring of Phe5 side chain (S…C.ar) and the three phenyl rings interact tighter with the side chains of residues Leu2, His6 (Rcen = 5.80 Å, $\theta = 75^{o}$) and Phe23 (Rcen = 5.27 Å, $\theta = 49^{o}$) (see Table 4). The interaction between the oxygen atom of the amide group and the calcium ion (O…Ca^{2+}) is also more favourable (see Table 4).

Decrease of the carbon chain length between the amide group and the aromatic ring R^1

Molecular structure **3** (see Table 3) was derived by decreasing the carbon chain length between the amide group and the aromatic ring of the R^1 chain. The higher binding energy of this analogue shows that the R^1 chain is accommodated inside the active site by FlexX in a more favourable way when the number of carbon atoms is decreased by two carbon atoms. Thus, a tighter interaction of the sulphur atom with the edge of the aromatic ring of Phe5 side chain (S…C.ar) is observed along with a better accommodation of "Phenyl2" and "Phenyl3" rings which interact more tightly with the side chains of Leu2 and Phe23 (Rcen = 5.23 Å, $\theta = 50^{o}$) (see Table 4). The interaction between the oxygen atom of the amide group and the calcium ion (O…Ca^{2+}) (see Table 4) is also tighter. The carboxylate group interacts with calcium ion (O…Ca^{2+}) and Gly31 (O…H–N) more favourably, in comparison with FPL67047XX inhibitor (see Table 4).

Insertion of substituents in the para-position of the aromatic ring R^1

Insertion of substituents in the para-position of the aromatic ring R^1 leads to molecular structures **4**, **5** and **6**. These analogues indicate more favourable binding energies than the FPL67047XX inhibitor but **6** shows the most favourable binding (see Table 3). This may be attributed to the participation of the hydroxyl group in a hydrogen bond with the oxygen atom of Leu2 (1.93 Å, O–H…O=C) which contributes to a higher binding energy in the GIIA sPLA$_2$ active site (Fig. 3). Hence, the rest of the critical key interactions become more favourable (see Table 4). The hydroxyl group is also able to establish van der Waals contacts in the binding cavity and therefore it is believed that this increases the binding energy. The high binding energy of molecular structure **6** is proposed that might have higher binding inhibitory activity than FPL67047XX.

In the case of molecular structure **4** (see Table 3), the methoxy group participates in aromatic/aliphatic interactions with the side chain of His6 (4.26 Å, between the carbon atom of methoxy group and the ring's centroid of imidazole group). The "tilted-T" aromatic (π–π) stacking interaction between the "Phenyl1" ring and the side chain of His6 is more favourable (Rcen = 5.79 Å, $\theta = 73^{o}$, see Table 4). More favourable are also the interactions of the carboxylate group with the calcium ion (O…Ca^{2+}) and Gly31 (O…H–N) (see Table 4). The interaction of the

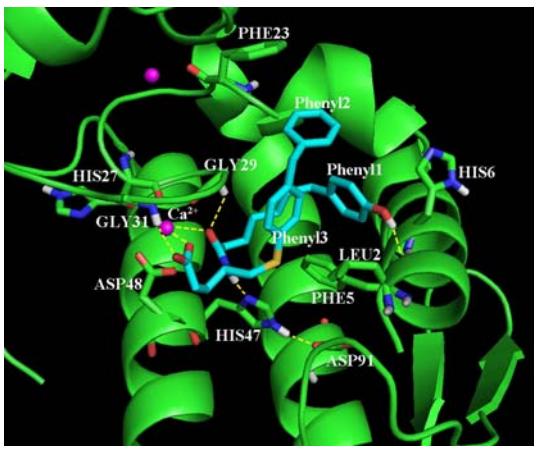


Fig. 3 The binding mode of molecular structure **6** at GIIA sPLA$_2$ active site as predicted by FlexX

Table 4 Distances of the main interactions measured in angstrom (Å) as they were calculated by FlexX

	1 (FPL67047XX)	2	3	4	5	6	7	8	9
Ca^{2+}–O (–COOH, –SO$_3$H, –PO$_3$H$_2$)	2.18	2.09	1. 68	2.04	1.71	2.11	2.27	2.00	1.78
Ca^{2+}–CONH	3.31	3.21	1.95	3. 42	3.71	2.60	3.67	4.19	3.27
His47–NH	2.12	2.12	2. 46	2.16	1.92	1.92	1.98	1.81	1.92
Gly29-CONH	1.63	1.73	2. 46	1.71	2.08	1.60	1.84	1.82	1.56
GlY31-O (–COOH,–SO$_3$H,–PO$_3$H$_2$)	2.79	2.03	1. 92	2.28	2.11	2.08	2.29	2.04	2.25
Phe5-S	5.65	5.30	4. 37	4.07	4.50	4.44	4.15	3.69	3.98
His6-Phenyl1 (Rcen)	6.01	5.80	6. 10	5.79	5.76	5.62	5.66	6.14	5.95
Phe23-Phenyl2 (Rcen)	5.46	5.27	5.23	5.59	5.50	5.04	5.62	5.66	5.30
Leu2-Phenyl3	3.52	3.25	3. 43	3.84	4.08	3.50	4.20	3.99	3.20

sulphur atom with the edge of the aromatic ring of Phe5 side chain (S…C.ar) (see Table 4) is also tighter.

The binding energy of molecular structure **5** (see Table 3) is more favoured than the binding energy of the FPL67047XX inhibitor, but less favoured than the binding energy of molecular structures **4** and **6**. The nitro group of the R^1 participates in a hydrogen bond with a water molecule near the GIIA $sPLA_2$ active site. That is, the reason the binding energy increases in comparison with the FPL67047XX inhibitor. On the other hand, the nitro group cannot participate in hydrogen bonds or in aromatic/aliphatic interactions with the residues of the active site, as the hydroxyl and methoxy groups. That explains the reason why the molecular structure **5** shows lower binding energy than molecular structures **4** and **6**.

Bioisosterism

Bioisosteric replacements have been widely used in the drug design [61, 62]. The bioisosteric groups of carboxylate, namely phosphonate and sulphonate, replace it in the molecular structures **7** and **8**. More favourable binding energy was shown by molecular structure **8** in comparison to molecular structure **7** (see Table 3). The oxygen atom of the sulphonate group indicates a tighter interaction with calcium ion (O…Ca^{2+}) than the carboxylate and phosphonate groups (see Table 4). In addition, the oxygen atom of the sulphonate group interacts more favourably with Gly31 (O…H–N) than the oxygen atoms of the carboxylate and phosphonate groups (see Table 4). In the past, other GIIA $sPLA_2$ inhibitors have been successfully modified by replacing the carboxylate group with bioisosteric groups and especially the phosphonate group [20, 51] because it emulates the interactions of phospholipids' phosphate group in the *sn*-3 position with GIIA $sPLA_2$, as the catalytic mechanism proposed [57].

The molecular structure **9** is characterized by a chiral centre of absolute (*R*)-configuration, four carbons in the R^1 chain, a hydroxyl group as a substituent in the para-position of the phenyl ring in R^1 chain and a sulphonate group instead of the carboxylate group. Molecular structure **9** indicates the highest binding energy than all the other analogues presented in this study (see Table 3; Fig 4). Furthermore, all the critical key interactions of molecular structure **9** are more favourable than the initial FPL67047XX inhibitor (see Table 4).

The conclusions following from the design, methods and results are: (1) the (*R*)-enantiomer presents higher binding energy than the (*S*)-FPL67047XX inhibitor; (2) the decrease of the number of carbon atoms of the R^1 chain increases the binding energy; (3) the insertion of a hydroxyl group in the para-position of the aromatic ring of R^1 chain increases the binding energy; (4) the optimum number of carbon atoms in the amino acid part of the ligand is three; (5) the sulphonate group presents the most favourable interactions in comparison with carboxylate and phosphonate groups; and (6) the aromatic chains are essential for the binding activity because the phenyl rings participate in "tilted-T" aromatic (π–π) stacking and aromatic/aliphatic interactions.

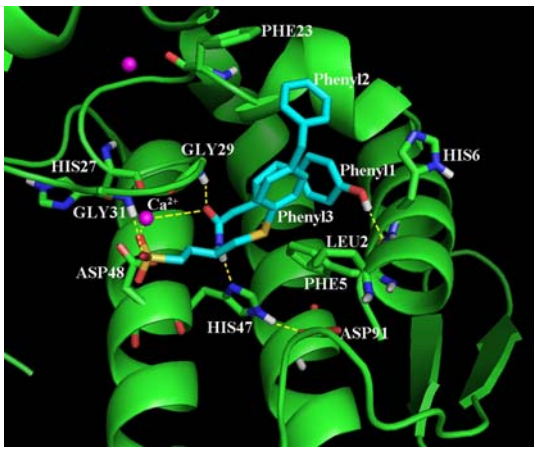


Fig. 4 The binding mode of molecular structure **9** at GIIA $sPLA_2$ active site as predicted by FlexX

In summary, molecular structures **6** and **9** have considerably 8.65 and 10.14 kJ/mol more negative binding energy, respectively, than the initial FPL67047XX inhibitor. These rationally designed structures can be synthetic targets for the development of potentially more active inhibitors of GIIA $sPLA_2$.

Conclusion

The aim of this study is to design new analogues with enhanced binding energy in the GIIA $sPLA_2$ active site by systematic modifications of the pharmacophore segments of the FPL67047XX inhibitor. Using inhibitors co-crystallised with the enzyme, the FlexX docking program was tested and found suitable to reproduce experimental crystallographic X-ray data. The newly designed analogues were then docked in the GIIA $sPLA_2$ active site.

The results extracted by the docking calculations of the newly designed analogues led to the understanding of the stereo-electronic factors affecting the binding energy. By decreasing and increasing the carbon atoms between the amide group and carboxylate group, the optimum number of carbon atoms between the two groups becomes three. Replacing the aromatic system with aliphatic chains reveals that the aromatic system is very essential due to its ability to participate in aromatic (π–π) stacking interactions. The (*R*)-enantiomer exhibited higher binding energy than the (*S*)-FPL67047XX showing more favourable

interactions in the GIIA $sPLA_2$ active site because the first one shows better binding and better accommodation of the pharmacophores in the enzyme active site. Insertion of a hydroxyl substitution in the para-position of the "Phenyl1" ring led to higher binding energy due to its ability to participate in hydrogen bonds and might be in van der Waals contacts with the enzyme binding cavity. Replacement of the carboxylate group with the bioisosteric sulphonate group led to better binding energy because the sulphonate group can better emulate the interactions of the phospholipids' *sn*-3 phosphate group proposed by the catalytic mechanism.

Comparisons of the interactions, of those analogues with the interactions of the initial FPL67047XX inhibitor contributed to the understanding of stereo-electronic factors that enhance the binding energy. The results of those calculations navigated the design of molecular structure **9** by modifying the FPL67047XX inhibitor using all the structural modifications that have shown higher binding energy. Molecular structure **9** indicated the highest binding energy than all the other analogues present in this study.

The FPL67047XX inhibitor can be considered as a starting structure for the GIIA $sPLA_2$ lead optimization because it can be rationally modified to improve the binding energy. We propose molecular structures **6** and **9** as potential synthetic targets for the development of GIIA $sPLA_2$ inhibitors with higher binding energy. It is up to medicinal chemists to take advantage of these theoretical calculations and synthesize potentially more active GIIA $sPLA_2$ inhibitors.

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