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ORIGINAL PAPER

Interactions of selected indole derivatives with phospholipase A_2: *in silico and in vitro* analysis

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Abstract Phospholipase A2 (PLA_2) is one of the key enzymes involved in the formation of inflammatory mediators. Inhibition of PLA_2 is considered to be one of the efficient methods to control inflammation. *In silico* docking studies of 160 selected indole derivatives performed against porcine pancreatic PLA_2 ($ppsPLA_2$) suggested that, CID2324681, CID8617 (indolebutyric acid or IBA), CID22097771 and CID802 (indoleacetic acid or IAA) exhibited highest binding energies. *In silico* analysis was carried out to predict some of the ADME properties. The binding potential of these compounds with human non pancreatic secretory PLA_2 ($hnpsPLA_2$) was determined using molecular docking studies. In order to corroborate the *in silico* results, enzyme kinetics and isothermal titration calorimetric analysis of the two selected compounds, IAA and IBA were performed against $ppsPLA_2$. From the analysis, it was concluded that IAA and IBA can act as competitive inhibitors to the enzyme and may be used as anti inflammatory agents.

Keywords Indoleacetic acid · Indolebutyric acid · ITC · Inducedfit docking · Phospholipase A_2

Introduction

PLA_2 (EC 3.1.1.4) is a lipolytic enzyme found in all cell types, including bacteria. PLA_2 is included in a family of disulfide-rich, Ca^{2+} dependent enzymes, which hydrolyzes the sn-2 position of glycero-phospholipids to release free fatty acids such as arachidonic acid and lysophospholipids [1]. Arachidonic acid is oxygenated further into a variety of products which modulate the inflammatory reactions [2]. Hence it is considered that the enzyme initiate the formation of inflammatory mediators such as prostaglandins, leukotrienes, platelet-aggregation factors and lysolipids [3, 4]. The excess production of inflammatory mediators leads to pathological conditions such as rheumatoid arthritis, bronchial asthma, ulcerative colitis, SLE, psoriasis, and Crohn's disease. Inhibitors of PLA_2 may act as anti-inflammatory agents and investigation on them may help to design better anti inflammatory compounds [5].

The active site of PLA_2 is a well defined hydrophobic channel. A histidine and aspartic acid residues together with a water molecule located in the active site act as the catalytic machinery in PLA_2. An oxyanion hole is found to stabilize the transition state after nucleophilic attack in PLA_2 by the backbone NH of Gly30 assisted by the charge of the Ca^{2+} ion [6, 7]. It is assumed that any compound which blocks the active site channel or masks histidine or aspartic acid residues located in the active site can act as a PLA_2 inhibitor. Many natural and synthetic compounds have been reported as PLA_2 inhibitors. Natural compounds like aristolochic acid [8], atropine [9], eugenol [10], berberine [11] and catechol [12] inhibit enzyme activity by binding in the active site.

Some of the indole derivatives have been reported in the treatment of inflammation. In 1995, researchers at Lilly pharmaceutical company have designed novel indole derivatives as strong inhibitors of $sPLA_2$ by computer aided molecular design [13]. Indole-3-glyoxamide derivatives have also been designed to act as human PLA_2 inhibitors based on the structure activity relationships [14]. Indole derivative, varespladib was reported as a selective human PLA_2 inhibitor [15]. Several other indole derivatives such as indolizine, indene, indoxam and bis indoles have also been designed and studied as PLA_2 inhibitors [16–18].

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In the present study, molecular docking of selected indole derivatives has been carried out in order to test their ability to act as PLA_2 inhibitors. Enzyme kinetic and isothermal titration calorimetric (ITC) analyses were also carried out to validate the *in silico* results.

Materials and methods

Selection of ligands and ADME property prediction

Around 160 indole derivatives were randomly selected and downloaded from the PubChem database (http://pubchem.ncbi.nlm.nih.gov/). Screening of the compounds has been done based on the Lipinski's rule of five [19]. Further the selected ligands were prepared and optimized for docking by LigPrep module of Schrödinger program using MMFF force field [20]. Maximum possible number of conformers for each ligand was generated with various ionization states, tautomerism, stereochemistry and ring conformations. All the generated conformers were saved as a single data set for the docking studies.

Important physico chemical properties such as molecular weight (mol_wt), number of hydrogen bond donors (HBD) and acceptors (HBA), van der Waals surface area of polar nitrogen and oxygen atoms (PSA) and solvent accessible surface area (SASA) with a probe radius 1.4 Å were calculated using QikProp module of the Schrödinger program. Other relevant properties such as IC_{50} value for blockage of HERG K + channels (QPlogHERG), apparent Caco-2 cell permeability (QPPCaco) and MDCK cell permeability (QPPMDCK), binding to human serum albumin (QPlogKhsa), percentage of human oral absorption (%HOA) were also predicted using QikProp module.

Protein preparation for docking studies

The atomic coordinates of porcine pancreatic PLA_2 (ppPLA2) in complex with catechol (PDB ID: 3O4M) [12] and human non pancreatic secretory PLA_2 ($hnpsPLA_2$) in complex with 1-benzyl-5-methoxy-2-methyl-1h-indol-3-yl-acetic acid (indole 3) (PDB ID: 1DCY) [21] were downloaded from Protein Data Bank (http://www.pdb.org). The protein structures were prepared using protein preparation wizard of Schrödinger program. Water molecules in the crystal structure were deleted and the polar hydrogens were added to the protein. The structure was minimized by applying OPLS-2005 force field [22, 23]. The minimization process was terminated when the root mean square deviation (RMSD) of the minimized structure relative to the crystal structure exceeded 0.30 Å.

Docking analysis

A grid of dimension 12 Å was set with the crystallographic ligand as the center of the grid. The residues Phe 5, Arg 6, Ile 9, Leu 19, Phe 22, Asn 23, Tyr 28, Gly 30, Gly 32, Asp 49 and Tyr 69 were in the selected grid. The prepared ligands in the data set were docked to the active site of PLA_2 by XP docking method.

The best scored compounds after XP docking were selected and induced fit docking (IFD) was carried out using the program Schrödinger. The IFD protocol allows flexibility to both the receptor and ligand. Initially the protein was kept as rigid. Top 20 poses per ligands were then docked by applying flexibility to protein residues which are within 5 Å from the ligand using Prime module. Glide score was calculated and the best poses were selected based on it.

IFD analysis of the selected compounds was done with the $hnpsPLA_2$ also to understand the mode of interaction. The protein was prepared based on the same protocol as mentioned above. The best docked poses were selected based on the glide score.

Enzyme kinetics studies

Among the top scored compounds, IAA and IBA were cheaply available hence their inhibitory potentialities were analyzed. The $ppPLA_2$, IAA and IBA were purchased from Sigma Aldrich, Bengaluru, India. The enzyme inhibition assay was carried out using the reported protocol [12] with 0.07 nM PLA_2 and 1.4 nM inhibitor concentration. Lineweaver–Burk plots were drawn, and Michaelis constant (K_m) and maximal velocity (V_{max}) were determined from the plot.

Isothermal titration calorimetric assay

Approximately 0.01 mM solution of PLA_2 was prepared in deionized water and 0.2 mM solutions of IAA and IBA were prepared in water containing 0.01 % NaOH. Both protein and ligand samples were degassed before loading to the ITC machine.

The calorimetric titrations were performed at the temperature 298.15 K using VP-ITC isothermal titration calorimeter from Microcal (Northampton, MA, USA) as described in the manufacturer's instruction manual. The 5 μl of ligand solutions were added from the rotating syringe to the cell which contains the enzyme solution. Five seconds was taken for each injection and a time interval of 120 s was set between two consecutive injections to allow the exothermic peak resulting from the reaction to return to the baseline. Total 56 injections were made. The reference power was set as 10 μcal and the stirring speed was adjusted to 307 rpm.

Table 1 Physico-chemical properties predicted for the selected ligands using QikProp program

Ligand	mol_MW (130–725)	HBD (0–6)	HBA (2–20)	QPlogPo/w (−2–6.5)	SASA (300–1000)	PSA (7–200)	Volume (500–2000)	QPlogHERG (concern < −5)	%HOA (>80 % is high, <25 % is poor)	QPlogKhsa (−1.5–+1.5)
CID2324681	243	2	2	2.8	411	60	687	−2.0	85	−0.24
CID8617	203	2	2	2.6	449	65	730	−2.7	80	−0.2
CID2097771	305	2	2	2.4	391	63	624	−1.9	80	−0.36
CID802	175	2	2	1.9	383	65	610	−2.2	76	−0.4

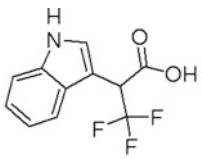
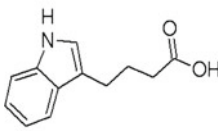
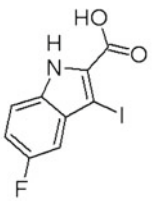
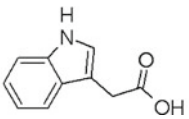
The volume of the first injection was 2 μl. The heat changes between PLA_2 and 0.01 % NaOH solution was subtracted from the original value and the final data at the end of the injections was fitted by a nonlinear least square method using ORIGIN software from Microcal. The binding constant (K), enthalpy change (ΔH), entropy change (ΔS) and binding free energy (ΔG) were calculated.

Results and discussion

Among the 160 selected compounds, 134 ligands satisfied Lipinski's rule of five and these were subjected to XP docking for the screening purpose. Their glide scores were in the range of −2.37 to −9.51 kcal mol^{-1}. Six compounds having glide scores better than −8.00 kcal mol^{-1} were taken for IFD. The compounds selected for IFD analysis were CID19417986, CID2324681, CID8617, CID40426947, CID22097771, CID802 and their glide scores were −9.51,−9.42,−9.19,−8.77,−8.52,−8.36 kcal mol^{-1} respectively. Based on the IFD results, four top scored compounds such as CID2324681, CID8617, CID22097771, CID802 were taken for the detailed *in silico* analysis of ADME properties.

About 45 pharmacologically relevant properties of these compounds were predicted and some of the properties of these componds are given in Table 1. Physico chemical properties such as PSA, molecular volume, SASA, OA, %

Table 2 Intermolecular interactions and glide scores of the selected ligands with $ppsPLA_2$

No	Compound Name	Structure	No of H bonds	No of vdW contacts (≤ 4Å)	G Score in kcal mol^{-1} with $ppPLA_2$
1	CID2324681		2	79	-11.67
2	CID8617		1	67	-11.10
3	CID22097771		0	58	-10.73
4	CID802		2	31	-9.80

of HOA, QPLogKhsa and IC_{50} on QPlogHERG were found in the allowed range. All compounds were found to be CNS inactive and their ability to cross the blood brain barrier was also very low.

The schematic representation of the ligands and their binding parameters with $ppsPLA_2$ were shown in Table 2. Among the four compounds, CID2324681 showed the highest glide score (−11.67 kcal mol^{-1}). Binding of the compound is stabilized by two hydrogen bonds with the enzyme. The N atom present in the indole ring makes a hydrogen bond with O atom of Phe 22. Also the O atom of the carboxyl group makes a hydrogen bond with N atom of Gly 30. The carboxylic group makes two coordinate bonds with the Ca^{2+} ion, which is important for the catalytic activity of PLA_2. The ligand also makes 79 van der Waals contacts (≤ 4 Å) with the surrounding protein atoms.

In the case of CID8617, the O atom of the carboxyl group makes a hydrogen bond with N atom of Gly 30 and the carboxyl group of the ligand makes two coordinate bonds with the catalytic Ca^{2+} ion. Sixty seven van der Waals contact were also found to stabilize the binding of CID8617 in the active site of the enzyme. The glide score obtained was −11.10 kcal mol^{-1}.

The binding of CID22097771 in the active site of enzyme was stabilized by coordinate bonds and van der Waals contacts. No hydrogen bonds were observed between the ligand and the enzyme. It was observed that the carboxyl group of

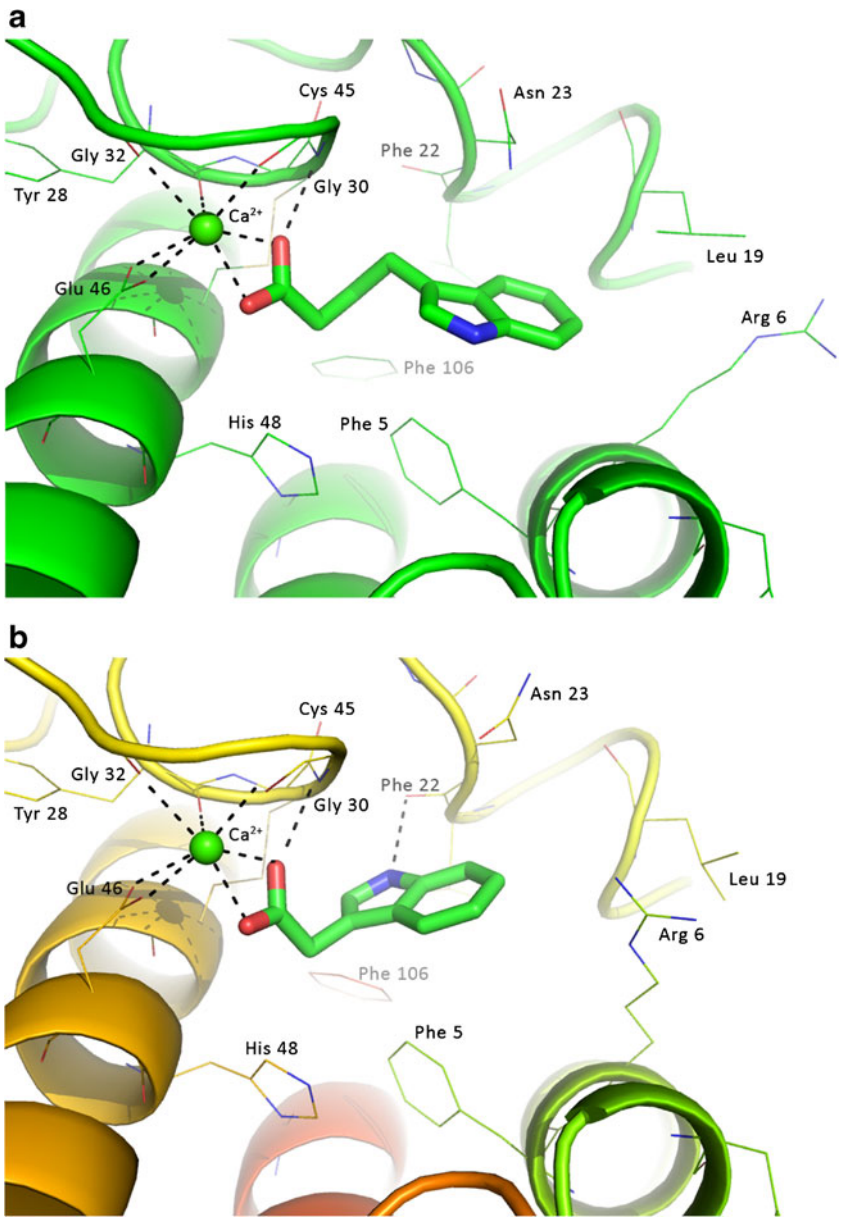


Fig. 1 Mode of binding of IBA (**a**) and IAA (**b**) in the active site of PLA_2. Hydrogen bonds are shown by doted lines

the CID22097771 was involved in the coordinate bond with Ca^{2+}. There are 58 van der Waals contacts between the ligand and the protein.

In the case of CID802, two hydrogen bonds were seen with protein residues along with two coordinate bonds. As explained in the case of CID2324681, the N atom present in the indole ring and O atom of the carboxyl group were involved in hydrogen bonds with O atom of Phe 22 and N atom of Gly 30 respectively. Thirty one van der Waals contacts were also seen between the ligand and the enzyme residues. The binding details of these four compounds with $ppsPLA_2$ have been given in Table 2.

The mode of binding and affinity of these ligands to $hnpsPLA_2$ were also studied and compared with some known inhibitors. The highest glide score (−10.38 kcal mol^{-1}) was found for CID8617. The glide scores for CID2324681, CID802 and CID22097771 were −9.74, -9.46 and −9.01 kcal mol^{-1} respectively. Indole-3, 4-(1-benzyl-3-carbamoylmethyl-2-methyl-1h- indol-5-yloxy)-butyric acid (indole-6) and 3-(1-benzyl-3-carbamoylmethyl-2-methyl-1h-indol-5-yloxy)-propyl-]-phosphonicacid (indole-8) have been reported to limit $hnpsPLA_2$ activity by blocking its active site. Hence these compounds were also docked into the active site of $hnpsPLA_2$ and compared with the binding affinity of the ligands under study. The indole-3, indole-6 and indole-8 bind in the active site with a glide scores −5.39, −5.31 and −5.69 kcal mol^{-1} respectively. From the comparison, it was found that the compounds under study could bind to the $hnpsPLA_2$ with much better binding energies than the reported inhibitors. Also the observations showed that these ligands can act as PLA_2 inhibitors.

The PLA_2s from porcine, bovine and venom shares high structural similarities with $hnpsPLA_2$. The active site residues and the position of the catalytic Ca^{2+} ions are arranged more or less in the same manner among all PLA_2s. It was identified that the top scored compounds CID8617 and CID802 are IAA and IBA respectively and they are known as auxins. The mode of binding of these two compounds in the active site of $ppsPLA_2$ has been displayed in the Fig. 1a and b respectively. Since these compounds are mainly known for their plant growth hormonal activities, it is interesting to test whether these compounds have any function relevant to human health.

As the compounds IAA and IBA were available to us, the enzyme kinetics and ITC analysis with these compounds were carried out. From the enzyme kinetics studies, it was found that both compounds inhibit $ppsPLA_2$ in a competitive manner and among these IBA inhibits more strongly than IAA. The K_m value for native PLA_2, inhibited PLA_2 with IBA and IAA were 1.33, 2.5 and 1.67 nM respectively. The V_{max} was found to be the same (i.e., 7.14 nM/ml/min) (Fig. 2). The inhibition constants (K_i) for IBA and IAA are 1.59 nM and 5.48 nM respectively. Similarly the IC_{50} value calculated using Cheng–Prusoff equation is 1.793 μM for IBA and 6.19 μM for IAA.

The IC_{50} values of some known indole compounds such as (1-benzyl-5-methoxy-2-methyl-1H-indol-3-yl)acetic acid [24], 2-(1-benzyl-5-methoxy-2-methyl-1H-indol-3-yl) acetamide [25] and 2-(1-benzyl-2-ethyl-5-methoxy-1H-indol-3-yl) acetamide [26] were 13.6, 0.84 and 0.26 μM respectively. The IC_{50} value of IBA was comparable to that of other known indole derivatives.

The result of ITC analysis of IAA and IBA toward $ppsPLA_2$ at 303 K is shown in Fig. 3a and b respectively. A total of 58 injections were made and non linear least square fitting method was applied to interpret the data. The binding constant (K), change in enthalpy (ΔH), change

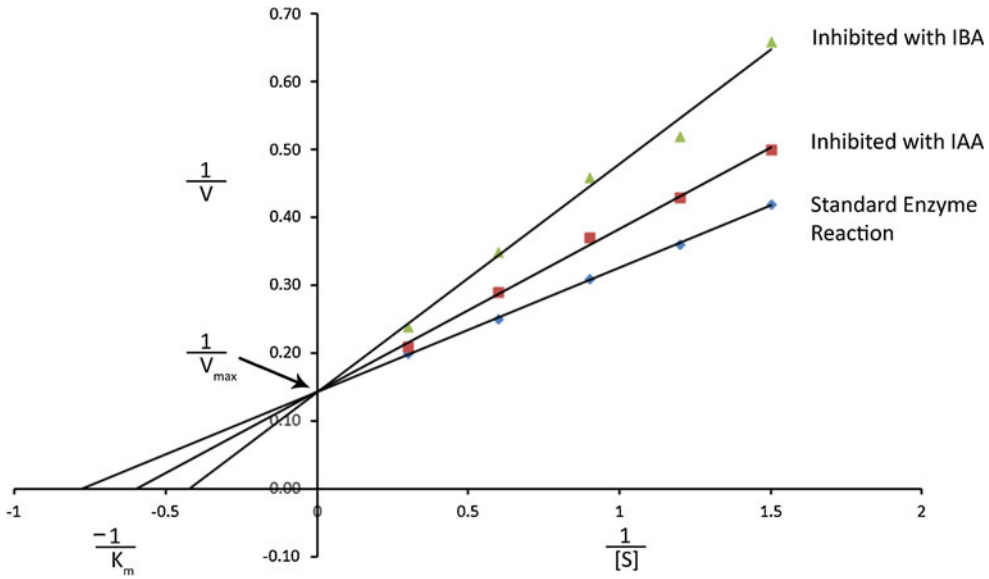


Fig. 2 Lineweaver-Burk plots of inhibition by IBA and IAA

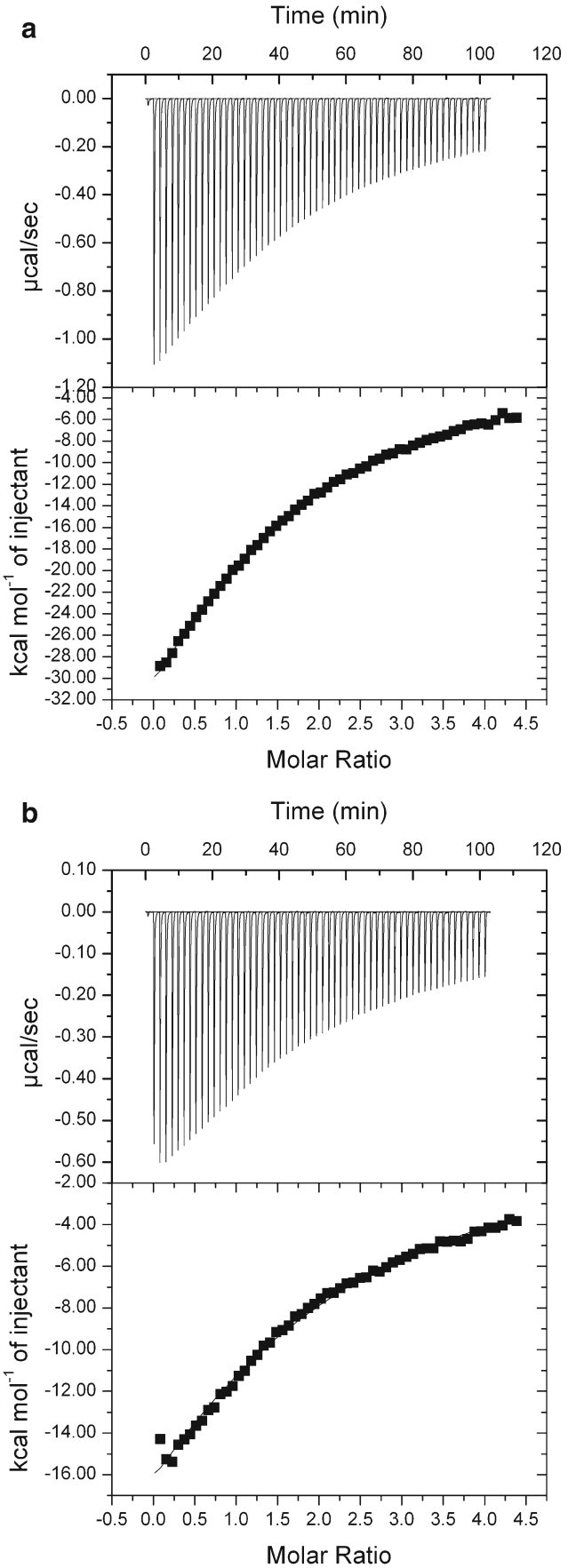


Fig. 3 Isothermal titration calorimetric analysis of PLA_2 with IBA (**a**) and IAA (**b**). The curve represents the non-linear least-squares fit of the energy released as a function of the compounds added during the titration. Raw thermal power signal (*top*) and plot of integrated heat versus ligand/protein molar ratio (*bottom*)

in entropy (ΔS) and binding free energy (ΔG) were calculated and is shown in Table 3. The isothermal curves of both IAA and IBA was fitted with $n=1$. The negative ΔG value indicates that the binding of each compound in the active site is thermodynamically favorable. The binding of these two ligands were enthalpy driven with negative enthalpy value. The binding energy obtained for these two ligands are −6.29 and −6.08 kcal mol^{-1}. The higher binding constant obtained for IBA from the ITC analysis probably explains the greater affinity of IBA toward PLA_2 than IAA. From the result, it was concluded that the IBA and IAA can effectively bind to the active site of PLA_2.

Conclusions

From the binding studies, it was concluded that all the discussed compounds such as CID 2324681, CID 22097771, IBA and IAA effectively bind to the active site of PLA_2 in such a manner that they can mask the catalytically active Ca^{2+} ion and the key active site residues responsible for the catalytic activity. Also it was found that all compounds exhibit the physicochemical properties suitable to make them drug lead compounds.

Using the program Qikprop, drugs already in use were screened in order to find the ones structurally similar to the compound under study. Five drugs, which have more than 85 % structural similarity were selected and they were Riluzole, Narpoxen, Carprofen, Diflunisal and Clonidine. All five drugs are known for their anti inflammatory protperties [27–31].

The enzyme kinetic and ITC analysis also points to the effective binding of IAA and IBA against PLA_2. The structural modification of these two compounds may enhance its binding affinity and thereby better non steroidal anti inflammatory compounds may be designed. The study also shows that compounds like IAA and IBA, which are known as plant growth hormones may have applications on animal systems.

Table 3 Thermodynamic parameters calculated for the binding of IBA and IAA to PLA_2 from ITC analysis

Sl No	Compound	N	K mol^{-1}	T (K)	ΔS cal mol^{-1} deg	ΔH cal mol^{-1}	ΔG in kcal mol^{-1}
1							
1	IBA	1.24	4.28×10^4	298.15	−269	-8.649×10^4	−6.29
2	IAA	1.10	2.88×10^4	298.15	−209	-6.842×10^4	−6.08

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