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Inhibitory activities of selected benzoic acid derivatives against phospholipase A₂
Comparative studies on the inhibitory activities of selected benzoic acid
derivatives against secretory phospholipase A$_2$, a key enzyme involved in the
inflammatory pathway

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Abstract

Inflammation is considered to be a key factor of major diseases like cancer, Alzheimer’s disease, Parkinson’s disease etc. For the past few decades pharmaceutical companies are trying to explore new effective medications against inflammation. As a part of their detailed studies, lot of drug targets and drugs were introduced against inflammation. In this present study, the inhibiting capacity of selected benzoic acid derivatives like gallic acid, vannilic acid, syrinngic acid and protocatechuic acid against secretory Phospholipase A$_2$ (PLA$_2$), a major enzyme involved in the inflammatory pathways has been investigated. The detailed in vitro, biophysical and in silico studies carried out on these benzoic acid derivatives revealed that all the selected compounds have a uniform mode of binding in the active site of PLA$_2$ and inhibiting in micro molar concentrations. The study also focuses the non selective inhibitory activity of a NSAID aspirin against PLA$_2$.

Introduction

Secretory phospholipase A$_2$ (sPLA$_2$) is one of the oldest enzymes that was identified and studied. The enzyme was first purified and characterized in the 19$^{th}$ century from cobra venom. Later the presence of PLA$_2$ has been confirmed in the venoms of different organisms, pancreatic juices and exudates of inflammatory fluids etc$^1$. The significance of this enzyme has not diminished even in the 21$^{st}$ century as its new involvements were discovered in various life threatening diseases like diabetes, rheumatoid arthritis, asthma, autoimmune disorders, cancer and several neurological disorders$^{2-5}$. sPLA$_2$s are directly or indirectly involved in any of these diverse pathological processes by mediating the release of arachidonic acid (a potent inflammatory mediator)$^6$. The excessive production of arachidonic acid may leads to the production of other proinflammatory mediators such as prostaglandins, leukotrienes, thromboxane and platelet
activating factors. Hence the inhibition of PLA\textsubscript{2} is considered to be one of the important therapeutic approaches for the treatment of different inflammatory diseases since PLA\textsubscript{2} inhibitors can limit the production of inflammatory mediators like arachidonic acid.

The advantage of natural molecules over synthetic compounds in terms of side effects has been discussed over the years. For the same reason natural compounds has received a recognized reputation in the field of drug discovery. If the statistics of the reported inhibitors of sPLA\textsubscript{2} is taken in to account, a distinguishable balance can be visualized in the numbers of natural and synthetic inhibitor molecules. Among different categories of natural molecules, polyphenols are a group of plant secondary metabolites that are designated for plant’s defense mechanism. Studies suggested that almost all the plant phenolic compounds are evolved either from phenylalanine or shikimic acid. More than 8,000 polyphenolics have been reported so far from different plant species. Based on the number of phenol rings and associated functional groups, polyphenols are classified into different groups such as phenolic acids, flavonoids, stilbenes and lignans. Phenolic acids are further classified to derivatives of benzoic acid and cinnamic acid.

Due to their capacity to diminish oxidative stress, they are widely used for the treatment against various chronic diseases.

Gallic acid (GA) (3, 4, 5-trihydroxybenzoic acid), vanillic acid (VA) (4-hydroxy-3-methoxybenzoic acid), protocatechuic acid (PCA) (3, 4-Dihydroxybenzoic acid) and syringic acid (SA) (4-hydroxy-3, 5-dimethoxybenzoic acid) are four naturally occurring benzoic acid derivatives. GA is a plant phenol, abundantly found in vegetables, tea and red wines. The pharmacological activities of GA such as anti tumoral, anti allergic, anti ulcer activities has been explained earlier. GA and their derivatives were abundantly used in pharmaceuticals, cosmetics and for different cancer due to their high anti oxidant capacity. Chances of approving GA as
a drug candidate is high since it is non toxic even at higher concentrations. VA is an oxidized form of vanillin, widely used as a flavoring agent and is mainly found in the root of *angelica sinensis* (Chinese herb). The role of VA in Chinese traditional medicine has been discussed earlier. Some remarkable studies that are carried out on human peripheral blood mono nuclear cells and liver cells proved the adequate effect of VA. It is also found to be effective against the management of immune and inflammatory responses. Like GA, and VA, PCA is also seen widely in the edible plant kingdom. PCA is well known for its multifunctional activities like anti bacterial, anti cancer, anti ulcer, anti aging, anti fibrotic and anti diabetics etc. It exhibit anti inflammatory activities by limiting the activities of different enzymes involved in the inflammatory pathways. The snake venom PLA2 and lipoxygenease inhibitory activities of PCA have already been discussed. SA has received much attention in Chinese traditional medicines as it found abundantly in the Chinese medicinal plants. The potent pharmaceutical functions like anti oxidant, cholesterol lowering and nitrogen dioxide free radical scavenging activities were discussed for SA earlier. SA is also reported to be useful against various inflammatory disorders like arthrosclerosis, rheumatoid arthritis, thrombosis cancer etc. The anti microbial activity of SA against various micro organisms has also been reported.

In the present work, a comparative analysis on the effect of selected benzoic acid derivatives (figure 1) with PLA2 has been studied by means of *in vitro*, biophysical and *in silico* studies. The rationale behind the selection of benzoic acid derivatives are nothing but their known anti inflammatory properties and the structural similarities with the known inhibitors. The proposed work investigates the activity of a known widely studied NSAID ‘aspirin’ on PLA2.
Materials and Methods

In vitro studies

The activity of selected benzoic acid derivatives and aspirin on ppPLA$_2$ has been investigated by in vitro enzyme kinetics studies. The necessary chemicals including ppPLA$_2$, benzoic acid derivatives and aspirin were purchased from Sigma Aldrich, Bengaluru, India. The inhibitory activity of these compounds on ppPLA$_2$ was analyzed by colorimetric analysis$^{27}$. The enzyme solution of a concentration 0.07 µM was prepared in deionized water. Different concentrations (0.3 µM, 0.6 µM, 0.9 µM, 1.2 µM and 1.5 µM) of substrate soya lecithin were prepared in 10 mM CaCl$_2$ solution. Later a reaction mix was prepared by adding 50 µL of the enzyme and 1.38 ml of 500 mM Tris HCL buffer (pH 8.5) containing 10 mM CaCl$_2$ solution. Further the reaction mix was added to a solution containing 700 µl of deoxycholate, 50 µl of CaCl$_2$ and 50 µl of substrate solutions (in different concentrations). Then the complete mixture was incubated at 37°C for 5 min. During this time, lecithin will undergo rapid hydrolysis in the presence of PLA$_2$ and produce free fatty acids. 200 µL of this reaction mixture was taken and added to a mixture containing 14% NaOH (200 µl), 2 M hydroxylamine (200 µl) and ether (1.5 ml) to stop the reaction. Since the high pH generated in the presence of 14 % NaOH will alter the enzyme activity and the enzyme reaction will be stopped. The mixture was again incubated for 20 min at room temperature for the formation of hydroxamic acid derivatives. The solution was further mixed with 10% FeCl$_3$ and 3 N HCl (300 µl each) and optical density was measured at 570 nm. The same procedure was repeated in the presence of two different concentrations of benzoic acid derivatives and aspirin. The concentration of each ligand that are taken for the studies was 0.7 and 14 µM. After the experiment, reciprocal of optical density was plotted against the reciprocal
of the concentration of the substrates. From the plot *Michaelis constant* \( (K_m) \) and maximal velocity \( (V_{max}) \) were determined.

**Isothermal titration calorimetric analysis**

Thermodynamics of benzoic acid derivatives and aspirin upon binding to ppPLA\(_2\) has been investigated by isothermal titration calorimetric (ITC) analysis. The experiment was performed on a Microcal VP-ITC machine with an enzyme solution of concentration 0.01mM and ligand solutions of concentrations 0.2 mM respectively. All ligand solutions were prepared in 0.01 % DMSO as it is not dissolved in distilled water. At the same time the protein was prepared in double distilled water. Approximately 1.8 ml of the protein solution and 290 µl of ligand solutions were used for the experiments. Before loading the samples to the ITC machine, they were degassed properly in order to remove the trapped air. The parameters of ITC experiment are as follows, time interval between 2 consecutive injections was 180 seconds, reference power 10 µcal respectively. After the ITC experiments the binding isotherms were fitted using non linear least square fitting methods (implemented in Origin) and the following values like changes in enthalpy \( (\Delta H) \), changes in entropy \( (\Delta S) \), Changes in free energy \( (\Delta G) \), binding stoichiometry of ligand biding \( (n) \) and binding constants \( (K) \) were deduced. The effect of 0.01% DMSO on protein has identified and subtracted from original ITC curve to rule out the phony results raised by the presence of DMSO.

**Molecular docking**

The atomic level of interaction of selected benzoic acid derivatives and aspirin with ppPLA\(_2\) was investigated by Induced Fit Docking (IFD) methods. The receptor and ligand structures were downloaded from protein databank (PDB) and pubchem respectively. Even though high resolution structures of different sPLA\(_2\)s are available in PDB, the molecular docking studies
were performed with the crystal structure of porcine pancreatic phospholipase A₂ (ppPLA₂) in complex with 2-methoxy cyclohexa-2-5-diene-1,4-dione (PDB ID – 3HSW)\(^{28}\) since the crystal structure contain a complex ligand which is analogous to the selected benzoic acid derivatives. It was expected that the selected benzoic acid derivatives will also have similar kind of interaction in the active site of PLA₂ as observed in the selected receptor’s structure. Prior to molecular docking studies, the 3D structure of PLA₂ was prepared using OPLS 2005 force field with the help of protein preparation wizard available in Schrödinger suite. Protein preparation includes the following steps like deletion of all crystallographic water molecules, addition of hydrogen atoms to polar groups, correction of coordinate bonds to the metal ions and proper reorientation of disulfide bonds. After incorporating the necessary modifications/corrections the protein was minimized to some extend in order to approve the topological changes. The minimization was automatically stopped when the modified structure gets deviated a RMSD of 0.30 Å from its original structure.

Later, the downloaded structures of benzoic acid derivatives and aspirin were prepared with the help of MMFF force field using LigPrep module of Schrödinger suite. Ligand preparation includes following steps like optimization of geometry, generation of tautomeric structure at different pH levels etc. All the generated structures were then saved as a dataset and used for docking studies. The affinity of the ligands towards the active site of PLA₂ and the conformational changes induced to the protein residues by the ligands upon binding were investigated by IFD method. The prepared ligands and protein were used for the studies. In the IFD method, ligands and some of the selected protein residues were kept as flexible. The flexibility was determined by selecting the protein residues that are within 10 Å away from the crystallographic ligand. Constrained minimization of the receptor has been carried out at each
step of docking. Initially rigid docking of the ligands was carried out and best poses were retained based on the binding energy and the side chain flexibility. Prime module implemented in Schrödinger was used for applying flexibility and finally the best pose was selected on the basis of the glide score.

**Molecular dynamics**

In order to investigate the residence time of each ligand at the active site of PLA₂, 10 ns molecular dynamics simulation studies has been carried out using GROMACS 4.5 program package. The best docked poses corresponding to each ligand was taken as the initial structure for the MD simulation. Interactions with in the system were described with AMBER99 force field and GAFF with AM1-BCC charges using antechamber through the acype parser. The protonation states of both protein and the ligands were set as same as the one used for docking calculations. The whole system was solvated with approximately 13000 TIP3P water molecules and 2 number of Cl⁻ ions to neutralize the charge. The solvated system was minimized further and MD simulations were carried out using a time step of 2 fs, keeping all bonds involving hydrogen atoms frozen. Van der Waals interactions were evaluated using a cut off radius of 9 Å and the electrostatic part were computed using the PME method with a cut off radius of 9 Å. Simulations were performed in a constant volume/constant temperature ensemble (NVT). An equilibration phase for 1 ns was carried out using Berendsen thermostat (tT = 0.1). The production phase was carried out using Nose-Hoover algorithm (tT = 0.1), and the total simulations were extended up to 10 ns.

**Result and Discussion**

*In vitro enzyme kinetics studies*
The *in vitro* enzyme kinetics studies carried out in the presence and absence of inhibitors has revealed the rate of inhibition each compounds. The lineweaver burk plot suggests a competitive mode of inhibition of all studied compounds including aspirin. From the plot it was observed that the \( V_{max} \) remains unchanged in the presence and absence of the compounds and it is the indication of competitive enzyme reaction. It was also observed that the *michleis constant* (\( K_m \)) calculated for native enzyme reaction (in the absence of inhibitors) is low compared to the enzyme reaction that is carried out in the presence of inhibitors (at two concentrations). Since it is very difficult to represent the \( K_m \) of all ligands (at two different concentrations) in the same lineweaver burk plot, only one ligand concentration (14\( \mu \)M) was used to draw the lineweaver burk plot. The inhibition constants (\( K_i \)) and \( IC_{50} \) values of the selected compounds were calculated using Cheng–Prusoff equation. From the results it was understood that among the selected benzoic acid derivatives, GA, PCA and SA are three powerful compounds against PLA\(_2\) having \( IC_{50} \) value below 2\( \mu \)M. At the same time the other compound like VA is also found to be effective against PLA\(_2\) having an \( IC_{50} \) value is between 2 and 3 \( \mu \)M. Even though the known NSAID aspirin is non selectively inhibiting COX-2, it exhibit a promising affinity towards PLA\(_2\). The \( IC_{50} \) value obtained for aspirin is 8.742 \( \mu \)M. The complete details of enzyme kinetics results were showed in Table 1 and figure 2. Similarly the lineweaver Burk plot drawn at a concentration of 0.7\( \mu \)M were shown in supplementary data as figure a.

**Isothermal titration calorimetric studies**

The results of ITC experiment suggested that all the selected ligands are capable to bind to PLA\(_2\) in an effective manner. All binding isotherms are found to be exothermic in nature. From the stoichiometry value (\( n \)) it was understood that the selected ligands were interacting to PLA\(_2\) through a single binding site. Among the selected benzoic acid derivatives GA and PCA are
promising in terms of binding affinity. VA and aspirin are exhibiting comparatively weak affinity towards PLA$_2$. The binding free energies calculated from the binding isotherm explained that GA and PCA are exhibiting more affinity towards PLA$_2$ than the other selected benzoic acid derivatives. The complete details of binding of all the benzoic acid derivatives are shown in Table 2 and figure 3.

**Molecular modeling studies**

From the molecular docking studies, it was understood that all the selected benzoic acid derivatives exhibit a comparable docking score with aspirin. The docking scores obtained for GA, PCA, SA, VA and aspirin were -10.71, -10.68, -10.18, -8.97 and -10.16 kcal/mol respectively. The detailed analysis of the binding patterns revealed that all the selected benzoic acid derivatives are strong enough to binds at the doorway of PLA$_2$ active site cleft. It was also identified that the carboxylic acid of all the studied ligands are oriented towards the catalytic Ca$^{2+}$ and mediate a coordinate bonds with Ca$^{2+}$ atom.

The detailed analysis of binding of GA in the active site of PLA$_2$ revealed the presence of three hydrogen bonds. In which one is formed between the hydroxyl group located in the C3 position of GA and the O atom located in the backbone of Phe 22. It was observed that the hydroxyl groups at positions C4 and C5 mediate two hydrogen bonds with the NH2 atom of Arg 6. Apart from these a strong hydrophobic interactions were observed between Phe 22, Phe 5, Leu 31 and Leu 2 of PLA$_2$. Binding of PCA at the active site of PLA$_2$ is characterized by a single hydrogen bond formed between the hydroxyl group present at the C4 position of PCA and the NH2 atom of Arg 6. Apart from these, hydrophobic interactions formed between Phe 2, Phe 5, Leu 31 and PCA make it as a strong binder at the active site of PLA$_2$. In the case of SA, the presence of two methoxy group enhances the hydrophobic contacts with active site residues such as Phe 22, Phe
106, Phe 5, Leu 31 and Leu 2. It was also observed that two hydrogen bonds are present in the interaction of SA with PLA₂. Among them one is formed between the hydroxyl group present at C4 position of SA and NH2 atom of Arg 6. Other hydrogen bond was observed between the O atom located at the C5 position of methoxy group and ND2 atom of Asn 23. The interaction of VA with PLA₂ is mediated by one hydrogen bond and few hydrophobic interactions. The hydrogen bond was formed between the hydroxyl group present in the C4 position of VA and the the NH2 atom of Arg 6. Apart from these hydrophobic interactions with Phe5, Leu 2, Phe 22 and Leu 31 were also observed. The chemical structure of aspirin is similar to benzoic acid derivatives as it contains a benzoic acid moiety. Hence a similar mode of binding was observed with PLA₂. The oxygen atom present in the acetoxy functional group forms a hydrogen bond with Tyr 69. The hydrophobic interactions observed between the following residues like Phe 22, Phe 5, Leu 31 and aspirin were turned it as a stronger binder in the active site of PLA₂. The binding modes of all studied compounds with PLA2 are explained in figure 4. The distances of H-bonds between all ligands and proteins were shown in supplementary data as figure b.

The molecular dynamics simulations studies performed with the best docked complexes of PLA₂ and ligands revealed that the stability of ligands at their bound position. After the MD simulation studies, a graph were plotted by connecting RMSD vs time (figure 5). The RMSD has been plotted by superimposing different protein structures (extracted during the MD simulation at various steps) on the initial structure on the basis of their ligand positions. The studies revealed that all the studied ligands are stable at their bound positions. None of them were retracted from the binding positions during 10 ns MD simulations.
**Conclusion**

The anti-inflammatory activity of all the selected benzoic acid derivatives has already been discussed over the years. From the binding mode analysis it was concluded that all the ligands are exhibiting a similar mode of orientations in the active site of PLA$_2$ and are capable of masking the catalytic Ca$^{2+}$ through a series of coordinate bonds. It was also noticed that all the selected compounds and are strong enough to reduce the enzyme activity compared to the known NSAID aspirin. Among the selected benzoic acid derivatives, the order of PLA$_2$ inhibiting activity is as follows: GA > PCA > SA > VA and > aspirin. The studies also points that all the selected compounds have an impact on the major anti-inflammatory target PLA$_2$. Hence benzoic acid derivatives can be considered as a promising scaffold for the designing of better non-steroidal anti-inflammatory drugs.

**Acknowledgement**

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References


4) Dai Jin and J. Russell, Molecules 2010, 15, 7313-7352


Figure Legends

Figure 1 : Structural information of different benzoic acid derivatives selected for the studies

Figure 2 : The Line weaver-Burk plot of native PLA₂ and the enzyme inhibited with GA, PCA, SA, VA and AS

Figure 3 : Isothermal titration calorimetric analysis of PLA₂ with (A) GA, (B) PCA, (C) SA, (D) VA and (E) AS. The curve represents the nonlinear 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).

Figure 4 : Mode of binding of (A) GA, (B) PCA, (C) SA, (D) VA and (E) AS in the active site of PLA₂. Proteins residues are represented by thin lines and ligand molecule are represented by thick lines. Hydrogen bonds were also shown by dotted lines.

Figure 5 : A plot of RMSD versus time during 10 ns MD simulation of PLA₂ complexes with (A) GA, (B) PCA, (C) SA, (D) VA and (E) AS.
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Structural information of different benzoic acid derivatives selected for the studies
The Line weaver-Burk plot of native PLA2 and the enzyme inhibited with GA, PCA, SA, VA and AS

230x125mm (300 x 300 DPI)
Isothermal titration calorimetric analysis of PLA2 with (A) GA, (B) PCA, (C) SA, (D) VA and (E) AS. The curve represents the nonlinear 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).

230x198mm (300 x 300 DPI)
Mode of binding of (A) GA, (B) PCA, (C) SA, (D) VA and (E) AS in the active site of PLA2. Proteins residues are represented by thin lines and ligand molecule are represented by thick lines. Hydrogen bonds were also shown by dotted lines.

230x110mm (300 x 300 DPI)
A plot of RMSD versus time during 10 ns MD simulation of PLA2 complexes with (A) GA, (B) PCA, (C) SA, (D) VA and (E) AS.

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