JPP JOURNAL OF Pharmacy and Pharmacology
ROYAL PHARMACEUTICAL SOCIETY
JPP Journal of Pharmacy And Pharmacology
Research Paper

Ascorbic acid 6-palmitate: a potent inhibitor of human and soybean lipoxygenase-dependent lipid peroxidation

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Keywords
ascorbic acid 6-palmitate; human; lipid peroxidation; lipoxygenase; soybean

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Received April 2, 2013
Accepted November 16, 2013

doi: 10.1111/jphp.12200

Abstract

Objectives Lipoxygenases (LOX) are the key enzymes involved in the biosynthesis of leukotrienes and reactive oxygen species, which are implicated in pathophysiology of inflammatory disorders. This study was conducted to evaluate the inhibitory effect of water-soluble antioxidant ascorbic acid and its lipophilic derivative, ascorbic acid 6-palmitate (Vcpal) on polymorphonuclear lymphocyte 5-LOX and soybean 15-LOX (sLOX) *in vitro*.

Methods LOX activity was determined by measuring the end products, 5-hydroperoxy eicosatetraenoic acid (5-HETE) and lipid hydroperoxides, by spectrophotometric and high performance liquid chromatography methods. The substrate-dependent enzyme kinetics and docking studies were carried out to understand the nature of inhibition.

Key findings Vcpal potently inhibited 5-LOX when compared with its inhibitory effect on sLOX (IC50; 2.5 and 10.3 μM respectively, $P = 0.003$). Further, Vcpal inhibited 5-LOX more strongly than the known synthetic drugs: phenidone and nordihydroguaiaretic acid ($P = 0.0007$). Enzyme kinetic studies demonstrated Vcpal as a non-competitive reversible inhibitor of 5-LOX. In-silico molecular docking revealed high MolDock and Rerank score for Vcpal than ascorbic acid, complementing in-vitro results.

Conclusion Both in-vitro and docking studies demonstrated Vcpal but not ascorbic acid as a non-competitive inhibitor of 5-LOX- and sLOX-induced lipid peroxidation, suggesting a key role for lipophilic nature in bringing about inhibition.

Introduction

Lipoxygenases (LOXs) are a family of non-heme, iron-containing enzymes widely distributed among animals and plants. In mammals, three major types of LOX have been identified, namely 5-, 12- and 15-LOX, which insert dioxygen at the C5, C12 and C15 positions of arachidonic acid (AA), respectively.[1] 5-LOX, a key enzyme in the leukotriene (LTs) biosynthetic pathway, is responsible for the synthesis of LTB4, LTC4, LTD4, LTE4 and 5-hydroperoxy eicosatetraenoic acid (5-HETE) from AA.[2] These LTs formed due to 5-LOX activity are powerful mediators of inflammation and regulators of the immune system.[3]

Furthermore, the distribution of 5-LOX is essentially restricted to polymorphonuclear leukocytes (PMNLs), monocytes, macrophages, mast cells and B-lymphocytes. Therefore, LTs-mediated inflammation is most commonly associated with diseases such as asthma, atherosclerosis, chronic obstructive pulmonary disease, rheumatoid arthritis, cancer, liver fibrosis and Alzheimer's disease.[4–7] Additionally, 15-LOX is of particular interest since it can also oxidize the esterified fatty acids in biological membranes and lipoproteins, forming 15-hydroperoxy-eicosatetraenoic acid (15-HPETE) from AA and 13-hydroperoxy-octadecadienoic acid (13-HPODE) from linoleic acid.

Mammalian 5-LOX and 15-LOX have been implicated in other specific oxidative reactions relevant in the pathogenesis of atherosclerosis.[8,9] Alternatively, soybean lipoxygenase (sLOX) is a plant-derived 15-LOX that efficiently catalyses the oxidation of linoleic acid to 13-HPODE similar to mammalian 15-LOX. Based on the structural and functional similarities with mammalian LOXs, sLOX is commonly used for both mechanistic and inhibitory studies, and is widely accepted as a model for LOXs from other sources.[10,11]

During the biosynthesis of inflammatory mediators such as LTs, various reactive oxygen species (ROS) are generated as by-products. These free radicals augment the development of inflammatory reactions, along with production of pro-inflammatory LTs. The generated ROS also bring about lipid peroxidation, producing lipid peroxides and other cytotoxic lipid derivatives.[12] Thus, the lipid peroxides that are formed are highly reactive molecules, capable of altering the membrane fluidity, ion permeability, surface charge, membrane enzyme activity, cell signalling and can damage DNA, all of which alter the cellular functions. Such changes are assumed to be causative factors for ageing and are associated with several degenerative disorders.[2,12,13] In such conditions, the scavenging of free radicals, along with the inhibition of inflammatory LTs production through the inhibition of LOX pathways by single antioxidant molecule, will have a prospective therapeutic role in the treatment of a variety of inflammatory and allergic diseases.

Many natural and synthetic compounds with redox and non-redox potential are known to inhibit LOX enzymes.[14–16] Ascorbic acid is one of the most abundant, naturally occurring, water-soluble antioxidant that has been broadly studied for the treatment of many inflammatory disorders due to its effective antioxidant property, but its anti-inflammatory property is still controversial.[17–19] Further, ascorbic acid 6-palmitate (Vcpal), a synthetic hydrophobic ascorbic acid derivative with strong antioxidant property, is extensively studied for its hydrophobic property.[20–23] Since Vcpal is not naturally found in food sources, synthetic VC6P is used as a source of vitamin C and as an antioxidant food additive. In addition, it is also used as an antioxidant in processed foods like breads and peanuts as well as it has been used to increase the shelf life of vegetable oils and potato chips.[24–26] Previous studies have revealed that Vcpal prevents the peroxidation of low-density lipoprotein and protect human erythrocytes from oxidative damage.[27]

Interestingly, despite seemingly extensive characterization of the antioxidant effects of Vcpal, it is still unknown whether this compound could inhibit human 5-LOX and soybean 15-LOX. Therefore, we examined the inhibitory potential of ascorbic acid and its synthetic lipophilic derivative Vcpal using human 5-LOX and soybean LOX, the key enzymes involved in LTs-mediated inflammatory disease conditions. Further, docking studies were performed to determine the possible enzyme inhibitor interaction.

Materials and Methods

Chemicals

All the chemicals used were analytical grade. Ascorbic acid, ascorbic acid 6-palmitate (structure shown in Figure 1), adenosine triphosphate (ATP), dithiothreitol (DTT), AA, sLOX, linoleic acid, phenidone and nordihydroguaiaretic acid (NDGA) were obtained from Sigma Chemicals Co. (Saint Louis, MO, USA). Fatty acid was purchased from Sisco Research Laboratories Pvt. Ltd. (Mumbai, India). Solvents used for high performance liquid chromatography (HPLC) analysis were HPLC grade obtained from Ranbaxy Fine Chemicals Ltd (New Delhi, India).

Isolation of 5-lipoxygenase enzyme from polymorphonuclear leukocytes of human blood

Venous blood from healthy individuals who were not under any medication was collected into ethylene diamine tetraacetic acid (EDTA)-containing tubes. The protocols were reviewed by the Institutional Human Ethical Committee, University of Mysore (Sanction order No. IHEC-UOM No.40/Ph. D/2009-10). PMNL cells were isolated from

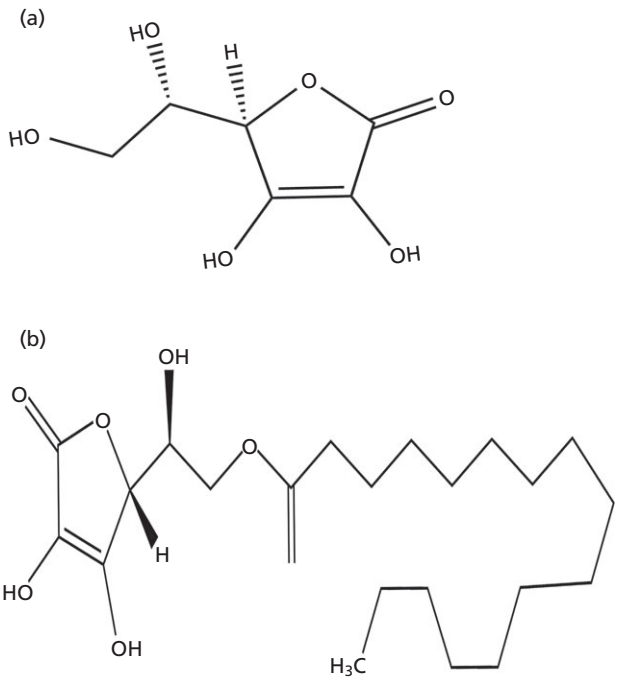

Figure 1 Chemical structure of ascorbic acid (a) and ascorbic acid 6-palmitate (Vcpal) (b).

blood by Ficoll-Histopaque density gradient and hypotonic lysis of erythrocytes.[28] All the procedures were performed at 4°C. PMNL cells were re-suspended in the phosphate buffer saline and sonicated for 20–30 s at 20 kHz to release the cytosolic 5-LOX enzyme into solution. This solution was centrifuged at 100 000*g* for 30 min at 4°C, and the supernatant obtained was directly used as enzyme source. Protein concentration was estimated by Lowry's method using fat-free bovine serum albumin as standard.

5-lipoxygenase enzyme assay

The enzyme assay was performed according to the method published by Aharony and Stein.[29] The standard reaction mixture for the 5-LOX assay contained 100 mM phosphate buffer pH 7.4, 50 μM of DDT, 200 μM of ATP, 300 μM of $CaCl_2$, 150 μM of AA and 5 μg of protein. Enzymatic reactions were carried out at room temperature. 5-LOX activity was measured as the amount of 5-HETE formed at 234 nm using Shimadzu 160A spectrophotometer (Tokyo, Japan). The molar extinction coefficient of 28 000/M/cm was used to calculate the specific activity of enzyme. The enzyme activity was expressed as μmol of 5-HETE formed/min/mg protein.

Soybean lipoxygenase enzyme assay

Enzymatic lipid peroxidation was measured spectrophotometrically following an increase in absorbance of lipid hydroperoxides formation at 234 nm.[30] The reaction medium contained 20 μM linoleic acid and 4 μg sLOX enzyme in 50 mM Tris buffer pH 7.4. The increase in absorbance at 234 nm was monitored by a Shimadzu UV 160A spectrophotometer. The molar extinction coefficient of 25 mM/cm was used to calculate the specific activity of enzyme. The enzyme activity was expressed as μmol of hydroperoxide formed/min/mg protein under the assay conditions.

Inhibitory studies with Vcpal on polymorphonuclear leukocyte 5-lipoxygenase and soybean 15-lipoxygenase

Vcpal, phenidone, palmitate and NDGA were dissolved in dimethyl sulfoxide (DMSO) at 2.0 mM concentration and pre-incubated with enzymes (human 5-LOX and sLOX) at different concentrations for 2.0 min, and then the reaction was initiated with addition of AA and linoleate separately. Controls with DMSO were maintained to check the effect of DMSO on lipid peroxidation reaction. The activity of 5-LOX and sLOX were measured as 5-HETE and lipid hydroperoxides formed at 234 nm spectrophotometrically, as described above. The IC_{min}, IC50 and IC100 concentrations were determined by Boltzmann dose response analysis.

Enzyme kinetic studies on inhibition of 5-lipoxygenase with Vcpal

The enzyme kinetics on inhibition of 5-LOX was studied using different concentrations of substrate AA (50 and 200 μM) vs IC25, IC50 and IC75 inhibitory concentrations of inhibitor. Double reciprocal plots of enzyme kinetics were constructed according to Lineweaver and Burk.[31]

HPLC separation of 5-lipoxygenase product 5-HETE and its inhibition by Vcpal

5-LOX product 5-HETE was resolved by HPLC method on a Luna straight phase silica column (2.5 mm × 25 cm). Enzyme assay was carried at room temperature as described above, in the presence and absence of Vcpal. The reaction mixture contained 100 mM phosphate buffer pH 7.4, 50 μM of DTT, 200 μM of ATP, 300 μM of $CaCl_2$, 150 μM of AA and 5 μg of protein. The reaction was stopped with the addition of 0.5 ml of 6N HCl at the end of 5 min. The 5-LOX product, namely 5-HETE, was extracted twice using organic solvent mixture of hexane and diethyl ether (1 : 1). The organic layers were pooled, dried under nitrogen and finally dissolved in 50 μl of mobile phase. The sample (10 μl) was injected on a straight phase silica column, and 5-HETE was resolved using a mobile phase of hexane : isopropanol : acetic acid (100 : 2 : 0.1) with a flow rate of 0.5 ml/min at 234 nm. The product 5-HETE formed was confirmed using an authentic 5-HETE standard obtained from Cayman Chemicals (Ann Arbor, MI, USA). The percent inhibition of 5-HETE levels was calculated from peak area of the control and inhibitor-treated sample.

Molecular docking

Molecular docking was carried out using Molegro Virtual Docker (MVD).[32] MVD is based on differential evaluation algorithm; docking scoring function, *E score* and the solution of algorithm, taking into account the sum of the intermolecular interaction energy between the ligand and the protein (E_{inter}), and the intramolecular energy of the ligand (E_{intra}). The structures of the ligands were constructed and optimized using the Dundee PRODRG2 Server[33] and saved in the MOL format. The crystal structure of soybean lipoxygenase-3 (protein data base (PDB) code: 1NO3) was obtained from the PDB database,[34] and it shares 63% sequence identity with human 5-LOX, which was retrieved using an automated docking MVD software platform, that is based on guided differential evolution and a force field-based screening function.[32] For the convenience of

docking, all water molecules and the inhibitor were excluded, whereas ferrous atom was retained in the active site, fixed to their crystal positions, throughout the docking process, necessary bonds, bond orders and hybridization, explicit hydrogens and protein charges were assigned and flexible torsion of ligands were detected. The Molegro docking wizard was used to simulate in-silico docking of ligands into sLOX using a MolDock scoring function of grid resolution of 0.30 Å, and search algorithm MolDock SE was used and a minimum of 10 independent runs per ligand were performed for better docking result. A value of population size and maximum interactions 100 and 10 000, respectively, were used for each run, and five best poses were retained for each ligand. Molecular visualization was done using Molegro Molecular Viewer 2008 version 1.2.0 (CLC bio, Cambridge, MA, USA).

Statistical analysis

The data are presented as specific activity of 5-LOX enzyme in the presence of different concentrations of inhibitors. The IC_{min}, IC25, IC50, IC75 and IC100 values were calculated by Boltzmann dose response analysis. Statistical differences were calculated by Duncan's multiple range tests at $P < 0.05$ using the SPSS statistical software (IBM Corp., Armonk, NY, USA). The results are presented as mean ± standard deviation of at least four determinations.

Results

The inhibitory effect of Vcpal on human PMNLs 5-LOX activity was evaluated by measuring the formation of 5-LOX product 5-HETE by spectrophotometric and HPLC methods. Vcpal inhibited PMNLs 5-LOX in a concentration-dependent manner, with IC_{min}, IC50 and IC100 value of 0.4 μM, 2.5 μM and 4.8 μM concentrations, respectively (Figure 2). The parent compound ascorbic acid failed to inhibit human PMNLs 5-LOX, whereas the inhibitory effect of palmitate was not significant when compared with Vcpal, even at higher concentrations tested. The IC50 value of Vcpal for inhibition of 5-LOX activity in comparison with synthetic 5-LOX inhibitors is presented in Table 1. Based on the IC50 values, the inhibitory effect of Vcpal on human 5-LOX was fivefold more potent compared with its effect on sLOX ($P = 0.003$). Further, Vcpal inhibited human 5-LOX 9.6 and 11.2-fold more strongly compared with known synthetic standard drugs phenidone and NDGA ($P = 0.0007$). This data demonstrate that 5-LOX has more affinity for Vcpal when compared with soybean 15-LOX enzyme. Furthermore, this data support Vcpal as a strong inhibitor of 5-LOX at physiological concentrations.

The inhibitory action of Vcpal on 5-LOX was further confirmed by HPLC method (Figure 3). The 5-LOX product 5-HETE was resolved on silica column by HPLC at 234 nm. 5-HETE is eluted as a single distinct peak at 7.88 min. Incubation of 5-LOX with different concentrations of Vcpal resulted in dose-dependent decrease in the levels of 5-HETE, as shown in Table 2. At 3.5 μM concentration, Vcpal exhibited 72 ± 5.0% inhibition.

Further, enzyme kinetic studies were carried out to understand the mechanism of inhibition of PMNLs 5-LOX by Vcpal. Substrate-dependent inhibitory activity was evaluated by varying concentrations of AA (50–200 μM) and different concentrations of Vcpal (IC25, IC50 and IC75). A double reciprocal plot of substrate vs enzyme velocity in the presence of Vcpal is presented in Figure 4. Vcpal treatment at 0, 1.6, 2.5 and 3.6 μM decreased the maximum velocity (Vmax) of the enzyme activity from 3.45 to 2.70, 2.15 and 1.65 μmol of 5-HETE/min/mg of protein, respectively, without much change in the Km value 112, 109, 114 and 112 μM (Table 3). The inhibitory constant

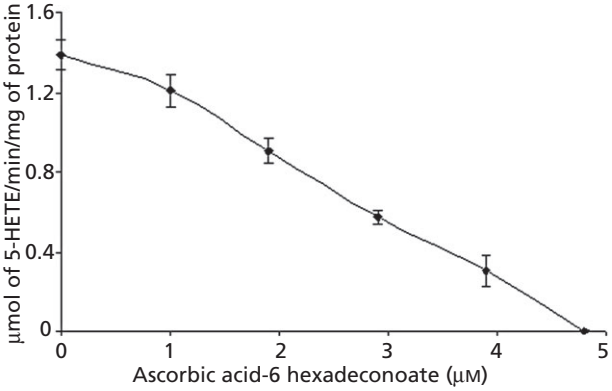

Figure 2 Dose-dependent inhibition of human polymorphonuclear leukocytes 5-lipoxygenase enzyme by Vcpal. Vcpal was incubated with 5-lipoxygenase enzyme isolated from human polymorphonuclear leukocytes for 2 min before starting the reaction by adding substrate. The enzyme activity was followed spectrophotometrically at 234 nm. The data are represented as mean ± standard deviation for four individual experiments.

Table 1 IC50 values of Vcpal for inhibition of human 5-lipoxygenase and soybean lipoxygenase activity in comparison with standard synthetic 5-lipoxygenase inhibitors

Compounds	Enzyme source	IC50 value[a] (μM)
Ascorbic acid	5-LOX/soybean LOX	00
Vcpal	5-LOX	2.5
Vcpal	Soybean LOX	10.3
Phenidone	5-LOX	24
NDGA	5-LOX	28

NDGA, nordihydroguaiaretic acid. [a]IC50 value is defined as the amount of inhibitor (μM) required to inhibit 50% of enzyme activity in the given reaction mixture. The linear regression analysis of the linear portion of the dose-dependent inhibition curve of each activity was used for the calculation of IC50.

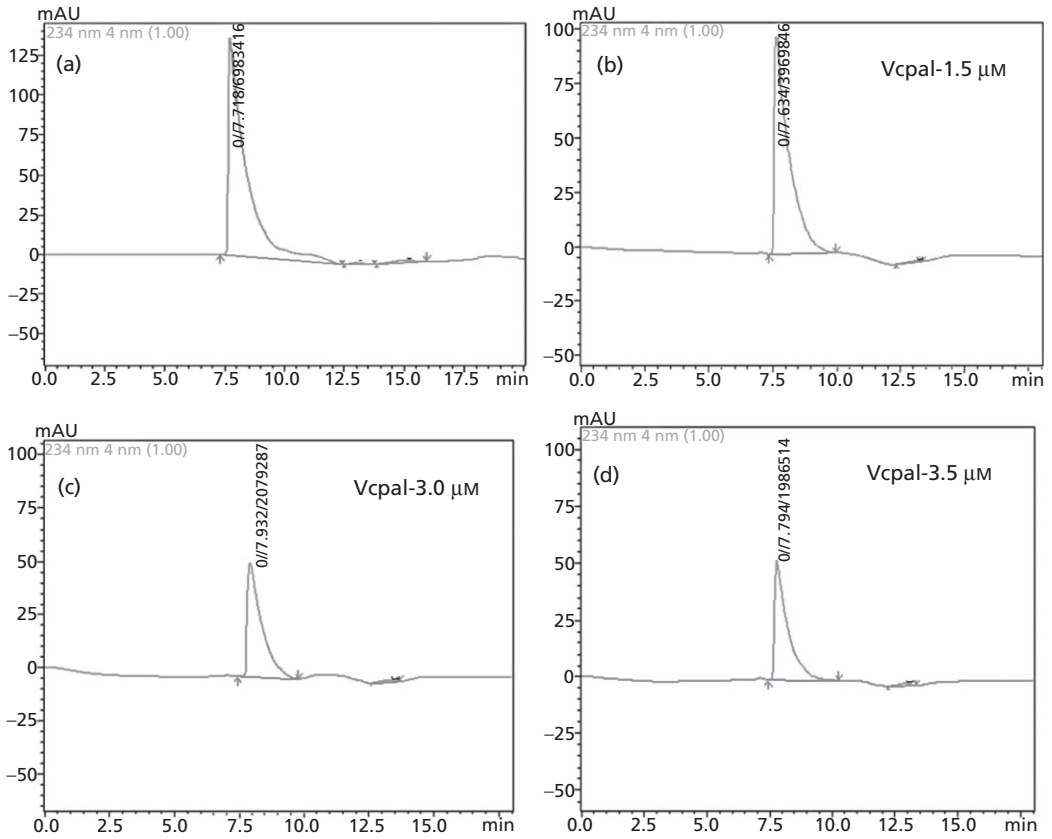

Figure 3 HPLC chromatograph of HETE resolved on silica column. A dose-dependent reduction in 5-HETE content was observed in the presence of Vcpal (b–d).

Table 2 Inhibitory effect of Vcpal on 5-lipoxygenase product 5-HETE content using HPLC method

Vcpal concentration (μM)	% inhibition compared with control
0.0	0.0
1.5	42.0 ± 3.53
3.0	70.6 ± 6.70
3.5	73.0 ± 3.35

K_i for Vcpal was found to be 0.37 μM. The enzyme kinetic data demonstrated that Vcpal is a non-competitive reversible inhibitor of human 5-LOX enzyme.

Lipid peroxidation consists of a radical-initiated reaction and can serve as suitable system for evaluating the antioxidant property. A number of LOX inhibitors are considered as antioxidant and are good inhibitors of lipid peroxidation. We further determined the effect of Vcpal and ascorbic acid on soybean 15-LOX-induced lipid peroxidation. Vcpal but not ascorbic acid significantly inhibited sLOX-dependent linoleic acid peroxidation by reducing hydroperoxide production with an IC_{min}, IC50 and IC100 value of 0.9 μM, 10.3 μM and 27 μM concentrations, respectively (Figure 5),

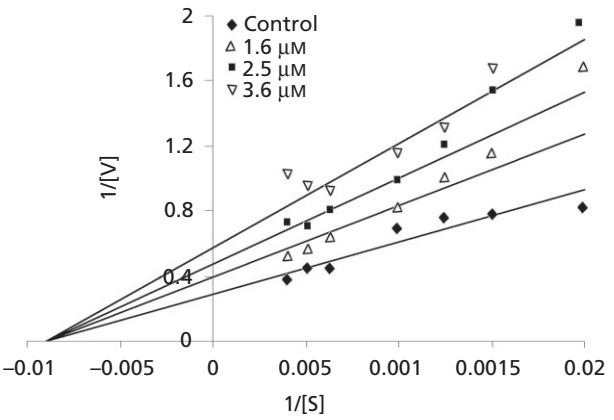

Figure 4 A double reciprocal plot for substrate-dependent inhibition kinetics of human polymorphonuclear leukocytes 5-lipoxygenase enzyme activity by Vcpal. 5-lipoxygenase enzyme was incubated with different concentrations of the inhibitor Vcpal in the presence of various concentrations of the substrate arachidonic acid. The changes in the Km values were not observed in the presence of different concentration of Vcpal.

Table 3 Effect of Vcpal on Vmax and Km valves of human polymorphonuclear leukocyte 5-lipoxygenase enzyme

Vcpal concentration (μM)	Km (μM)	Vmax (μmol of HETE/min/μg protein)
0.0	112.0	3.45
1.6	109.0	2.70
2.5	114.0	2.15
3.5	112.0	1.65

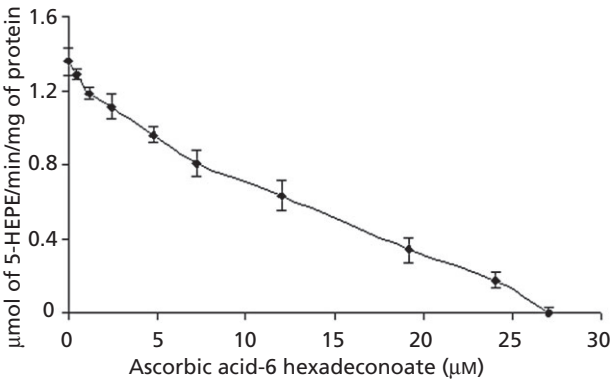

Figure 5 Dose-dependent inhibition of soybean lipoxygenase enzyme by Vcpal. Vcpal was incubated with soybean lipoxygenase enzyme for 2 min before starting the reaction by adding substrate. The enzyme activity was followed spectrophotometrically at 234 nm. The data are represented as mean ± standard deviation for four individual experiments.

while ascorbic acid failed to inhibit even at higher concentrations tested (Table 1). Comparison of dose linear inhibition of 5-LOX and 15-LOX by Vcpal elucidate that Vcpal is fivefold more potent inhibitor of human 5-LOX than soybean 15-LOX enzyme (Figure 2 and Figure 5).

Further, the docking studies were performed to determine the possible mechanism of action, as well as the differences in the specificity of the compound towards LOX enzymes. As shown in Figure 6a, the interaction between Vcpal and sLOX reveals a typical inhibitory mechanism for a potent inhibitor of 5-LOX, wherein its polar head and long hydrophobic tail play a crucial role in its better binding for the inhibition. The hydroxyl oxygen atoms present in the furan ring of Vcpal form hydrogen bonds with Arg386 (3.26 Å), Pro384 (2.75 Å), Asp382 (3.01 Å), Leu380 (2.93 Å) and Asp382 (2.90 Å). Additionally, the carboxyl oxygen of fatty acid backbone, which is involved in esterification linkage with ascorbic acid moiety, forms hydrogen bonds with Asp378 (3.20 Å and 3.11 Å). The hydrogen bond determines the best pose orientation for Vcpal in the binding site and stabilizes the hydrophobic interaction of its hydrophobic tail with surrounding amino acid residues Leu399, Ser387, Arg386, Pro423, Asp431, Val589 and Trp592, which reveal an efficient MolDock score and Rerank score when compared with standard inhibitors (Table 4). These docking scoring functions demonstrate the lead molecule Vcpal as a good inhibitor of LOX enzymes, whereas vitamin C fits into the hydrophilic core of the enzyme to form hydrogen bonds with surrounding amino acid residues. Figure 6b depicts the binding mode of ascorbic acid with sLOX. The oxygen atoms of ascorbic acid ring form hydrogen bonds with Val589 (2.63 Å), Asp592 (3.18 Å) and Trp593 (3.28 Å), Asn521 (3.31 Å, 2.78 Å and 2.73 Å), Gln598 (3.17 Å and 2.75 Å), Val594 (2.97 Å), Gln598 (2.86 Å and 2.75 Å), and Asp597 (2.78 Å). As shown in Figure 6c, the known standard inhibitor phenidone binds to the hydrophilic core of the enzyme, and forms hydrogen bonds with Asp375 (3.03 Å) and Ser517 (2.79 Å). MolDock score and Rerank score of acorbic acid, Vcpal and phenidone are given in Table 4.

Discussion

The PMNLs play an important role in the modulation of inflammatory responses. PMNLs are initially recruited at the site of injury and secrete elevated levels of leukotrienes, prostaglandins and ROS, which contribute to inflammatory pathophysiology.[1,13] Especially, LTs along with ROS accelerate the progression of the diseases by exacerbating the initial local inflammatory responses that include vasodilatation, local extravasation and leakage of blood vessels, eventually leading to tissue damage.[5–7,35] The production and release of LTs and ROS can be prevented by inhibition of LOX pathways by antioxidants and their derivatives. Ascorbic acid is one such naturally occurring antioxidant that directly reacts with aqueous peroxyl radicals and also indirectly co-stimulates the antioxidant properties of fat-soluble vitamin E.[17,18] Williams *et al.* demonstrated the inhibition of degranulation of PMNLs by ascorbic acid, leading to decreased release of myeloperoxidase, thus preventing potential tissue damage.[36] The earlier study also demonstrated the ability of ascorbic acid to inhibit the conversion of AA to PGF2α in guinea pig uterus.[37] Further, ascorbic acid also reduced the production of 12-hydoxyeicosatetraenoic acid, the major LOX product from human corneal tissue[38] confirming its interference in prostaglandin biosynthesis. Since ascorbic acid rapidly undergoes oxidation and irreversible degradation in the body,[37] many researchers reveal that antioxidant activity of ascorbic acid could be significantly enhanced by introducing a hydrocarbon tail to make it more stable and lipid-soluble.[23,39] Additionally, these synthetic derivatives are extensively studied for its antioxidant, antitumor and anti-inflammatory activity.[19–23,40–42] Ascorbic acid 6-palmitate (Vcpal), a synthetic lipophilic amphipathic derivative, is used as an antioxidant in food additives, vitamins, drug and dermatological products. The antioxidant potential of Vcpal

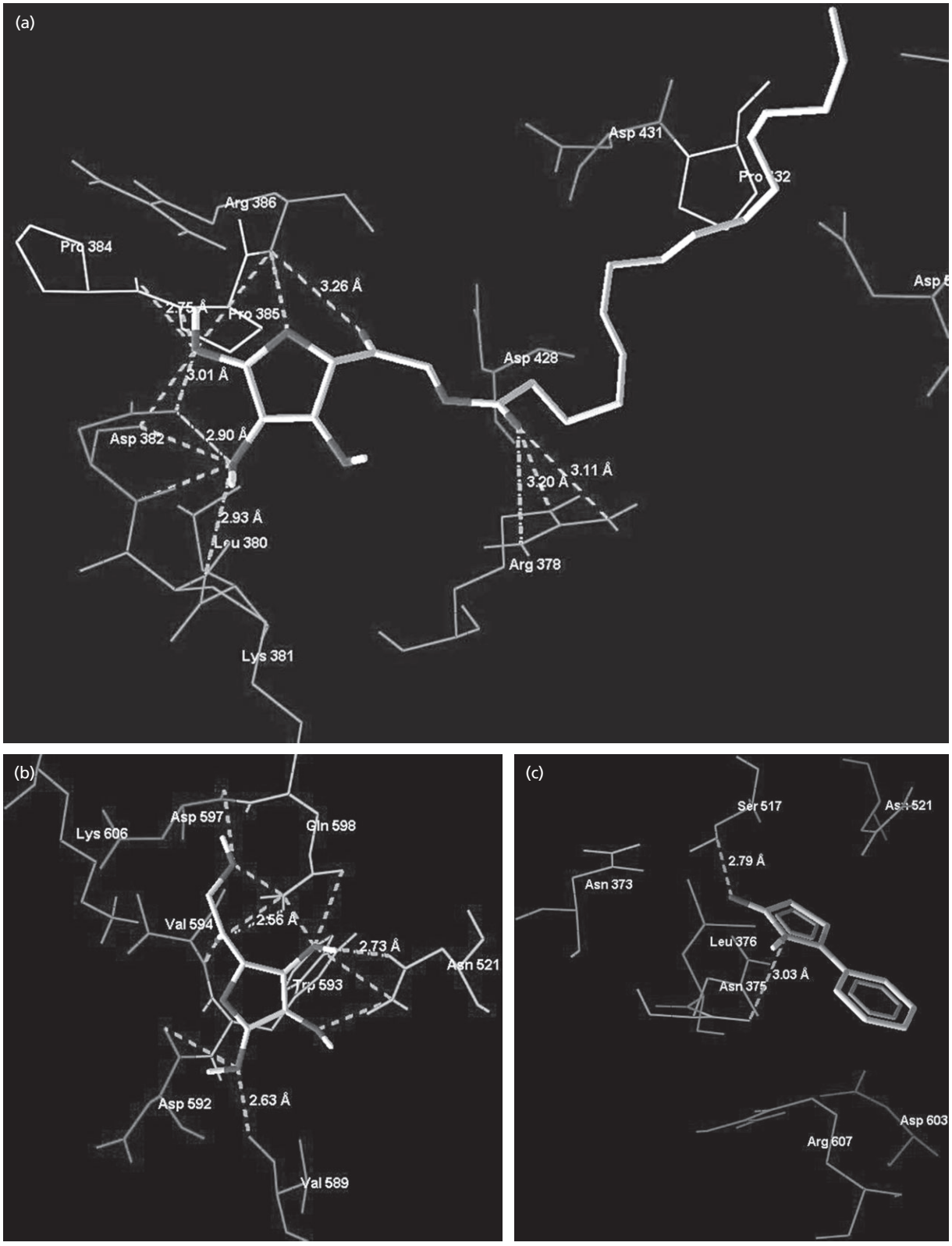

Figure 6 Schematic representation of hydrogen bond and hydrophobic interactions of soybean lipoxygenase with ascorbic acid (a), ascorbic acid-6 palmitate (b) and phenidone (c). Highlight the surrounding residues, electrostatic interaction and hydrogen bond interaction. Ligands are shown in stick model, and dotted line indicates hydrogen bond.

Table 4 Molecular docking results

Compound	MolDock score	Rerank score
Ascorbic acid	–83.20	–71.86
Vcpal	–137.29	–101.87
Phenidone	–74.375	–66.1619

The compounds were ranked on the basis of their MolDock and Rerank scores. The scores of the individual compounds are the best of five scores derived from independent docking rounds.

is attributed to the inhibition of glutathione s-transferase enzyme and neutralization of phospholipase A2-induced local toxicity.[43] However, the detailed mechanism and its interaction with human 5-LOX and soybean LOX are yet unknown.

In this study, we investigated the effect of ascorbic acid and Vcpal on inhibition of PMNLs 5-LOX and soybean LOX activity. Vcpal significantly inhibited PMNLs 5-LOX in a concentration-dependent manner with IC50 value of 2.5 μM and inhibitory constant (Ki) of 0.37 μM (Figure 2). In contrast, ascorbic acid did not inhibit the LOX enzymes. Further, the extent of 5-LOX inhibition by Vcpal was found to be tenfold higher than the standard synthetic inhibitors phenidone and NDGA ($P < 0.001$) (Table 1). The inhibition was further confirmed using the HPLC method, wherein incubation of 5-LOX with various concentrations of Vcpal resulted in dose-dependent decrease in the end product 5-HETE (73% inhibition at 3.5 μM (Figure 3 and Table 2)). Consistent with the above results, ascorbic acid did not inhibit the formation of 5-HETE. The above two results represent Vcpal as a potent candidate for therapeutic use in inflammation, with higher efficiency over the parent compound, ascorbic acid.

Ascorbic acid is a weak inhibitor of hyaluronidase and myeloperoxidases.[23,36] Report by Botzki demonstrated that Vcpal is a strong inhibitor of hyaluronidase activity compared with the parent compound, ascorbic acid.[23] The long alkyl chain of Vcpal appeared to interact with an extended, hydrophobic channel formed by mostly conserved amino acids of hyaluronidase enzyme.[23] The 5-LOX enzyme with hydrophobic channels or cavities helps in the conduction of electrons from substrate to the active site iron atom and allows a proper interaction with other hydrophobic compounds. This interaction is critical for the inhibition of LOX enzyme by hydrophobic compounds.[44] Our current observation further confirms that long hydrophobic alkyl chain of Vcpal is significant for the improved binding affinity of this molecule with LOX. These results are also in agreement with the observation made by Clapp *et al*., where hydrophobic thiols with enhanced hydrophobicity showed more effective inhibition of LOX activity.[45] Therefore, we infer that the side chain of Vcpal with additional hydrophobic residues interacts with the 5-LOX enzyme and enhances the inhibition of Vcpal compared with ascorbic acid.

The inhibitory mechanism of 5-LOX studied by AA- (50–200 μM) dependent enzyme kinetics revealed that Vcpal significantly did not change the Km value of 5-LOX (~112 ± 3 μM, $P = 0.08$) but decreased the maximum velocity (Vmax, 3.45 μmol of 5-HETE to 1.65 μmol, $P = 0.04$) of the enzyme, suggesting a competitive inhibition. These results also demonstrate that Vcpal, in addition to binding at the active site, may also interact with different sites of the enzyme. Supporting our observation, similar earlier report by Raghavenra *et al*. also demonstrated that eugenol at different concentrations did not change the Km value of 5-LOX but decreased the Vmax.[16] Furthermore, this suggests that antioxidant compounds like Eugenol and Vcpal can scavenge the lipid peroxyl radicals formed during the oxidation of AA, and thus block the formation of 5-HETE as evidenced by the decrease in Vmax during enzyme kinetics experiments (Figure 4).

Plant-derived soybean LOX (sLOX) is structurally and functionally similar to mammalian LOXs that efficiently catalyse the oxidation of linoleic acid to 13-HPODE. Therefore, sLOX is commonly used as a mammalian model molecule to study the mechanistic and inhibitory properties of human 12/15LOXs.[14,42] By virtue of their hydrophobic interaction with catalytic site, many n-alcohols as well as synthetic hydrophobic thiols inhibit sLOX.[45] Similarly in our study, Vcpal but not ascorbic acid significantly inhibits sLOX in a dose-dependent manner, with an IC50 of 13.4 μM concentration (Table 1, Figure 5). This data strongly confirm that the hydrophobicity of the inhibitor is essential for LOX inhibition.

The use of plant LOX to model mammalian LOXs will prove highly beneficial and supportive in structural characterization, mechanism elucidation and possibly the discovery of novel inhibitors of LOXs.[46] Due to unavailability of three-dimensional structures of 5-LOX, we used crystal structure of plant sLOX for our docking studies. Generally, the enzyme/inhibitor complexes are stabilized almost entirely by van der Waals, aromatic interactions in the hydrophobic region and by hydrogen bond in hydrophilic region.[32] Our docking study validates that Vcpal and ascorbic acid bind to the hydrophilic core of the enzyme and forms a hydrogen bond with surrounding amino acid residues. Vcpal had maximum interaction with high negative MolDock and Rerank scores, supporting the observed high inhibitory potency (2.5 μM for 5-LOX and 13 μM for sLOX), compared with ascorbic acid and phenidone (Table 4). This demonstrates the strong and specific binding of Vcpal into hydrophilic pocket of LOX enzyme.

Conclusion

This study demonstrates that Vcpal but not the parent compound L-ascorbic acid as a non-competitive inhibitor

of human PMNLs 5-LOX and sLOX induced lipid peroxidation. The docking studies correlate with observed in-vitro activity. The data suggest that the lipophilic nature of the compound plays a critical role in bring about LOX inhibition.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Acknowledgements and funding

We thank University of Mysore, Mysore for providing facilities to carry out this project. RM, ST and JMS acknowledge the Council of Scientific and Industrial Research (CSIR), New Delhi, India for CSIR-SRF.

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