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Research Article
Cis-parinaric Acid: A Non-redox Inhibitor of Lipoxygenase-1

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Abstract

Background and Objective: Cis-parinaric acid (CPA) is a naturally occurring polyunsaturated fatty acid found in the seeds of Makita tree. This chromophore is a C18 conjugated fatty acid fluoresces at 410 nm, when excited at 325 nm. Lipoxygenase (LOX) enzyme mediated reaction products are involved in inflammation. The objective of the work was to study the *in vitro* inhibition and interaction of soy LOX-1 with CPA. **Methodology:** Lipoxygenase-1 activity was performed by measuring the end products, hydroperoxy octadecadienoic acid using linoleic acid substrate in the presence or absence of CPA. The kinetics is deduced with varying concentration of substrate and inhibitor. The association constant for the binding of CPA to LOX-1 was determined by observing tryptophan quenching of the LOX-1 with the addition of CPA. **Results:** Cis-parinaric acid inhibits soy LOX-1 activity with an IC_{50} value of 18.8 μM. The mechanism of inhibition of soy LOX-1 by CPA is competitive with the Ki value of 9.8 μM. The binding constant for the binding of CPA to soy LOX-1 is $6.0 \pm 0.5 \times 10^4$ M^{-1}. CPA binds close to the iron cofactor of LOX with the distance of CPA to the iron atom being 3.3 Å. **Conclusion:** Kinetic and docking studies demonstrated that CPA is a non-redox type of inhibitor, inhibiting LOX-1 by competitive manner.

Key words: Conjugated fatty acid, fluorescence, binding constant, cis-parinaric acid, competitive inhibition, soy lipoxygenase

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Lipoxygenase mediates the stereoselective peroxygenation of the methylene interrupted unsaturated systems found in natural substrates like linoleic, linolenic and arachidonic acids[1]. Lipoxygenase catalyses the dioxygenation of polyunsaturated fatty acids containing 1,4-pentadiene system. The reaction products are chiral E, Z-conjugated hydroperoxy fatty acids having cytotoxic properties. In mammals, the products of LOX are involved in inflammation and immune response[2]. Due to the role of LOX products in inflammation and immediate hypersensitivity, there is much interest in inhibiting LOXs[3]. Analogues of linoleic acid and triple bond analogues of polyunsaturated fatty acids are known to inactivate LOX[4]. Oleyl sulfate binds tightly to the allosteric site and is an allosteric inhibitor of soy LOX-1 and human-15 LOX[5]. Mammalian LOXs are the target for drug design and since mammalian LOXs are difficult to purify, soy LOXs have been used as the template to design the inhibitors. The other advantage is, extensive structural and kinetic data on soy LOX-1 are available. Soy LOX-1 is used as a model, as the substrate specificity and inhibition characteristics are similar to mammalian LOX[6]. Interest in blocking the lipoxygenase pathway has driven interest in the discovery and research of compounds involved in inhibition of LOX[7].

Fatty acids with conjugated double bonds occurs naturally[8] and conjugated fatty acids are proposed to possess anti-atherosclerotic effect, to induce immune response and to modulate energy metabolism[9]. The other application of conjugated fatty acids is its use as fluorescent probe for studying the membrane structure[10]. The CPA is a fluorescent C18 fatty acid. Its fluorescent property is attributed to the presence of 4 conjugated π- electron bonds (Fig. 1). Sklar *et al.*[11] demonstrated that parinaric acid (9Z, 11E, 13E, 15Z octadecatetraenoic acid) can be used to detect phase transitions in bilayers and to study the interactions among lipids and proteins. The CPA can be incorporated into phospholipids by lipid biosynthetic pathways and spectroscopic investigations can be performed resulting with its wide use as a membrane probe[12]. Apart from its use as a probe CPA is reported to posses biological property. CPA is extracted from the plant seeds of Matika and the seed on consumption is toxic to humans. The CPA is reported to be cytotoxic to human leukemia cells in cell culture at concentrations of 5 μM or less. This is due to the sensitizing of tumor cells to lipid peroxidation, during which the free radicals generated react with electrons from cell membrane lipids, resulting in cell damage[13]. It is similarly cytotoxic to malignant gliomas grown in cell culture. Normal (non-tumorous) astrocytes grown in culture are far less sensitive to the cytotoxic effects of CPA[14]. This preferential toxicity towards tumor cells is due to a differential regulation of c-Jun N-terminal kinase and forkhead transcription factors in malignant and normal cells[15]. Owing to the similarity in the structure of LOX substrate and CPA, the present study was designed to evaluate the interaction and inhibition of LOX-1 by CPA *in vitro*. The report highlights the mode of inhibition of CPA by LOX. The enzyme kinetics is followed by molecular interaction studies to reveal the site and the strength of interaction. This is experimentally proven by fluorescence and molecular docking studies. Efforts are made to explore the potential of CPA as possible candidate for drug development.

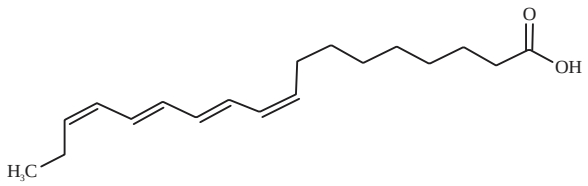

Fig. 1: Structure of cis-parinaric acid (CPA) $C_{18}H_{28}O_2$

MATERIALS AND METHODS

Cis-parinaric acid was obtained from Molecular Probes. Inc. (Junction city, OR, USA). All spectrophotometric measurements were made in a Shimadzu UV 1601 double beam spectrophotometer (Kyoto, Japan) using 1 cm path length quartz cells. Fluorescence measurements were made on a Shimadzu RF 5000 spectrofluorimeter (Shimadzu, Japan) attached to a circulating Peltier thermostat. A 10 mm path length quartz cell was used. The solution in the cuvette was stirred continuously with the help of Hellma cuv-o-stir® (Mulheim, Germany). All the measurements were carried out at 25°C, unless mentioned. Stock solution of CPA was prepared by dissolving CPA in absolute ethanol with constant purging of nitrogen. The concentration of CPA was calculated using $\varepsilon_{303\ nm} = 76000\ M^{-1}\ cm^{-1}$.

Assay of soy lipoxygenase-1: Soy LOX-1 was purified from defatted soy flour according to the method of Axelrod *et al.*[16]. The LOX-1 activity was determined by following the increase in absorbance at 234 nm due to the formation of hydroperoxide (product, $\varepsilon_{234\ nm} = 25000\ M^{-1}\ cm^{-1}$). The substrate is prepared according to the method of Axelrod *et al.*[16]. The amount of enzyme required to form 1 μM of hydroperoxide per min under the conditions of assay, was taken as one unit of activity.

Inhibition of soy lipoxygenase by cis-parinaric acid: For inhibition experiments, CPA were added in the concentration range 0-25 μM to 0.2 M borate buffer, pH 9.0, containing the enzyme and preincubated for 5 min. For the control no CPA was added. To the above, 100 mM linoleic acid was added and the reaction is followed for three minutes. The reaction was assayed by monitoring the formation of hydroperoxides at 234 nm using e = 25000 M^{-1} cm^{-1}. The percentage inhibition was calculated from the DOD at the end of three minutes. Kinetics of inhibition was analyzed by Lineweaver-Burk plot (L-B) and inhibition constant was calculated from the replot of (L-B) and Dixon plot[17]. The data obtained were fitted to a straight line by the least square method[17].

Fluorescence measurements of cis-parinaric acid with lipoxygenase-1: Association constant for the binding of CPA to LOX-1 was determined by following the intrinsic fluorescence quenching of LOX-1 with titration of CPA. The excitation wavelength was set to 295 nm, with the slit width of 5 and 10 nm, respectively. The concentration of LOX-1 was fixed to 0.37 mM. Approximately 1 mL aliquots of CPA were added from 2.75 mM stock in ethanol in the concentration range 0-11 mM. From the mass action plot the association constant for the binding of CPA to LOX-1 was calculated[18].

Docking studies: From the protein data bank (PDB ID. 1JNQ) the lipoxygenase coordinates with bound epigallocatechin (EGC)[19] was obtained. LOX-3 with EGC bound is selected for docking studies. Enzyme with bound ligand is preferable for the studying and docking of a new ligand as the side chains would be in suitable position. Molecular Virtual Docking Software (MVD 2010. 4. 0. 2) (Molegro Virtual Docker, Molegro ApS, Denmark) was used for computing, which performs the docking and calculations algorithm provided by the manufacturer was used to identify the binding cavities. Flexible docking was carried out and the program MolDock Score was used to evaluate. An exhaustive search for automated docking was set to perform by the dock engine with complete ligand flexibility to elucidate the mode of interactions. From pose clustering the possible binding conformations and orientations were analyzed.

Statistical analysis: Data are presented as Means±Standard Deviation. For all of the measurements, a minimum of three to four replicates was taken for data analysis. Using the commercially available software Origin 6.1 (Origin Lab, Northampton, MA, http://www.originlab.com), all of the values were averaged and plotted.

RESULTS

Due to the structural similarity with the fatty acid, the ability of CPA to inhibit LOX-1 is investigated. *In vitro* inhibition of LOX-1 with different concentration of CPA is used to follow the inhibition. The plot of absorbance at 234 nm versus the time period at which the reaction was followed in the concentration between 0-25 mM (Fig. 2a). With increasing concentration of CPA (0-25 mM), activity of LOX-1 decreased.

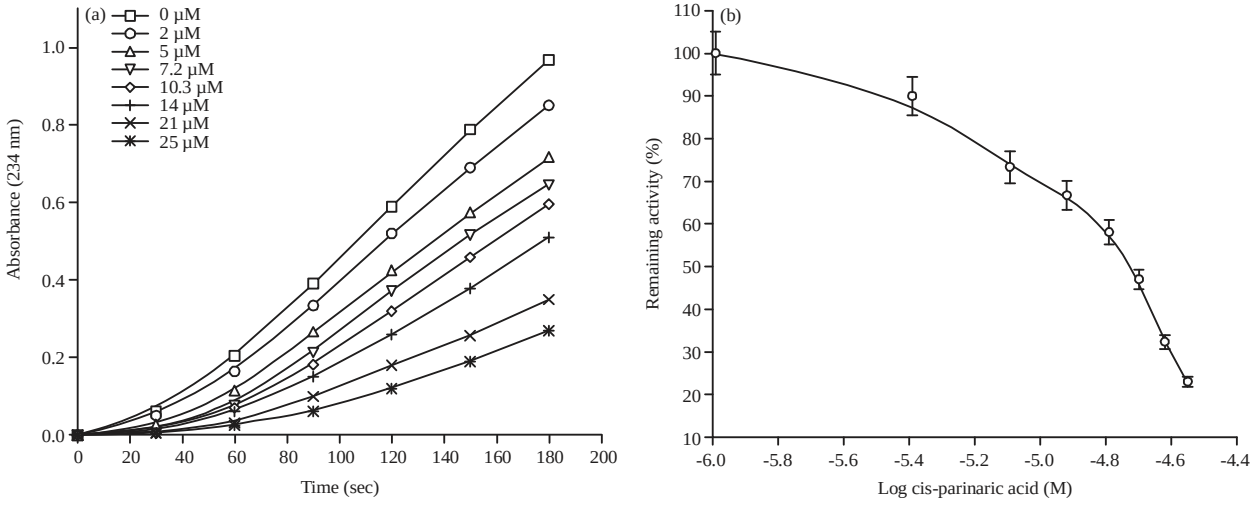

Fig. 2(a-b): (a) Time course of LOX-1 catalyzed reaction in the presence of CPA. Aliquots of CPA were added from CPA stock solution (6 mM in ethanol) to buffer containing LOX-1 and (b) Determination of IC_{50} value of CPA for inhibition of LOX-1

Values are average of three different sets of experiment

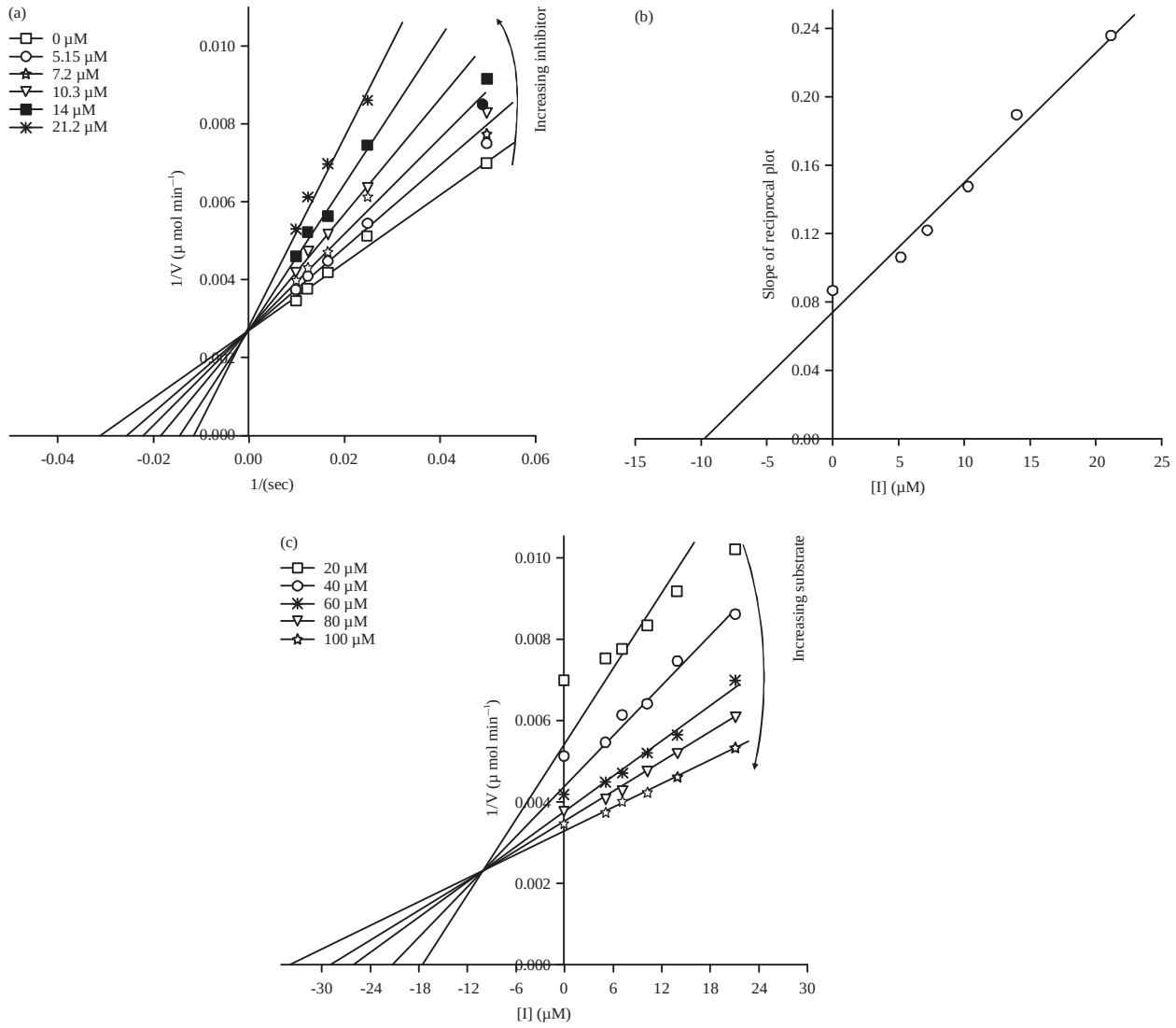

Fig. 3(a-c): (a) Lineweaver-Burk plot to determine the kinetics of inhibition of LOX-1 by CPA. The concentration of CPA varied from 20-100 mM, (b) Determination of Ki value of CPA and (c) Dixon plot
Values are average of three different sets of experiment

The residual activity of LOX-1 in the presence of CPA is represented in Fig. 2b. The concentration at which 50% of inhibition (IC_{50}) occurs is observed at 18.8 mM. Above 25 mM, CPA in buffer is insoluble and gets precipitated. The kinetics for inhibition of LOX-1 by CPA is investigated with varying concentration of substrate and inhibitor. Lineweaver-Burk (L-B) plot is represented in Fig. 3a. The L-B plot produce the lines intersecting on Y- axis (Fig. 3a) representing that the type of inhibition is competitive. The Ki value of CPA for inhibition of LOX-1 is 9.8 mM obtained from replot of Lineweaver-Burk plot (Fig. 3b). Steady state kinetics was analyzed from Dixon plot by plotting the inverse of velocity of reaction against different concentration of CPA (Fig. 3c) and the Ki agrees with the value obtained from L-B plot.

Plant and mammalian LOXs share 25% homology, while soy LOX-1 is 72% identical with LOX-3[19]. Highest sequence similarity is found in the catalytic domain containing non-heme Fe cofactor. Based on the experimental inhibition studies molecular docking studies were done which would provide insight on the nature and mode of binding of CPA to LOX. The binding of CPA to the ribbon model of LOX-3 (PDB ID-1JNQ) is shown in Fig. 4a. The closer observation reveals that CPA binds close to iron cofactor with the distance of carboxylate group of CPA to the iron being 3.3Å (Fig. 4b). The

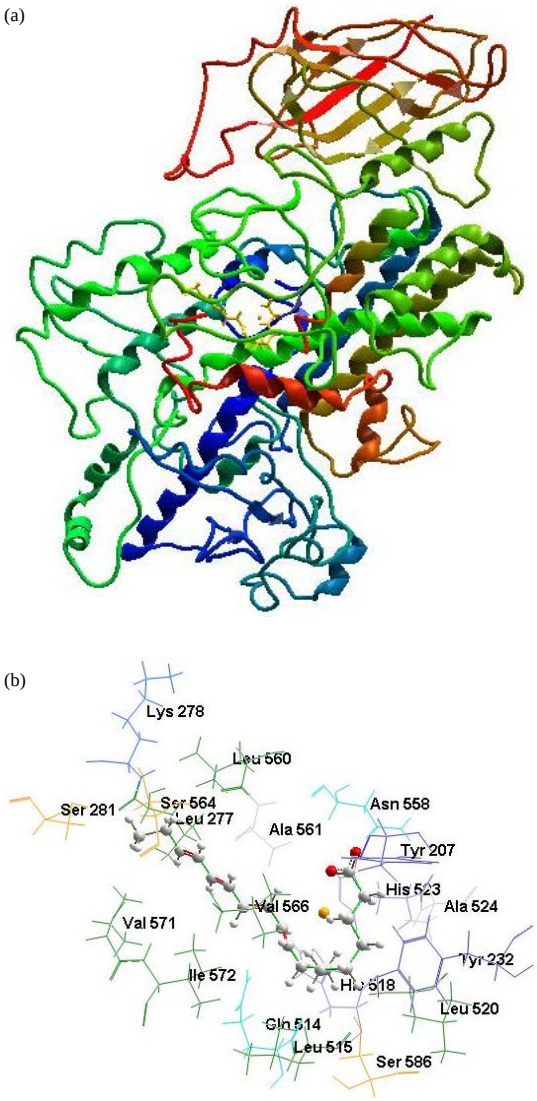

Fig. 4(a-b): Docking of CPA with lipoxygenase, (a) Cartoon ribbon model showing the binding of CPA to lipoxygenase-3 and (b) A closer look of the amino acid residues present at the binding site of CPA

ligand is occupying the site closer to the cofactor iron (enclosed in circle near Val 566). The amino acids closer to the CPA within 2.5 Å distance is represented in Fig. 4b. These are His518, His523, Ala524, Ala561, Ser564, Ser281, Tyr 217, Leu277, Leu560, Leu520, Leu515, Gln514, Val566 and Ile572. The CPA makes interaction with majority of the hydrophobic amino acids as revealed from Fig. 4b. Tyr 217 is closer to the oxygen atom of the carboxyl group of CPA. The substrates of LOX and polyunsaturated fatty acids interact with their olefinic part of the chain placed nearby to the iron and His 518[20]. In the presence of inhibitor, the substrate is inaccessible due to the preoccupation of CPA nearby the iron leading to the inhibition of the enzyme. Epigallocatechin (ECG) binds and inhibits LOX-3 by making interaction with the iron and nearby amino acids[19]. Degradation product of quercetin, protocatechuic acid is reported to bind nearby to the iron cofactor[21]. Tetrahydorcurcumin, the hydrogenated derivative of curcumin inhibits LOX by binding near the iron cofactor, thus inhibiting the enzyme[22]. Isoflavones from soy inhibits LOX by interacting and keeping the active site iron in the ferrous state[23].

The emission spectrum of LOX-1 and the LOX 1-CPA complex when excited at 295 nm are shown in Fig. 5a. In the absence of CPA, the fluorescence spectrum shows emission maxima of 333 nm due to the excitation of tryptophan residues in LOX-1. Titration of LOX-1 with CPA results in the quenching of tryptophan fluorescence at 333 nm, with the concurrent increase and shift in the emission maximum towards longer wavelength. Free or unbound CPA does not fluoresce at the excitation wavelength of 295 nm. Energy transfer was detected from the overlap of the tryptophan fluorescence emission spectrum of LOX-1 with the incremental addition of CPA. The red shift in wavelength observed with the addition of CPA is due to the resonance energy transfer from the tryptophan residues of LOX-1 to the bound CPA. There are 14 Trp residues in LOX-1 which woul account for the energy transfer leading to the shift in wavelength from 333-373 nm with the consecutive addition of CPA. The primary plot of quenching against varying concentration of CPA is shown in Fig. 5b and the inset is the mass action plot to calculate the association constant. The association constant is calculated by taking the values at 333 nm and the Ka is $6.0\pm0.5\times10^{4}$ M^{-1}. The trend in decreasing emission of tryptophan fluorescence and simultaneous increase in fluorescence of CPA at 333 and 373 nm, respectively is depicted in Fig. 5c.

DISCUSSION

The study describes the mechanism of inhibition of LOX-1 by CPA. Lipoxygenases (linoleate:oxygen oxidoreductase, EC 1. 13. 11. 12; LOXs) are a family of monomeric non-heme, non-sulfur iron dioxygenases, ubiquitous in both plants and animals. They catalyze the conversion of polyunsaturated fatty acids possessing cis, cis-1, 4-pentadiene unit into 1, 3-cis, trans-diene-5-hydroperoxides[3]. The LOX is implicated in many pathological conditions such as arthritis, psoriasis, prostate cancer[24] bronchial asthma and therefore inhibitors of LOX are useful in preventing the aggravation of inflammation. Nevertheless to say, many compounds are reported to inhibit LOX[25].

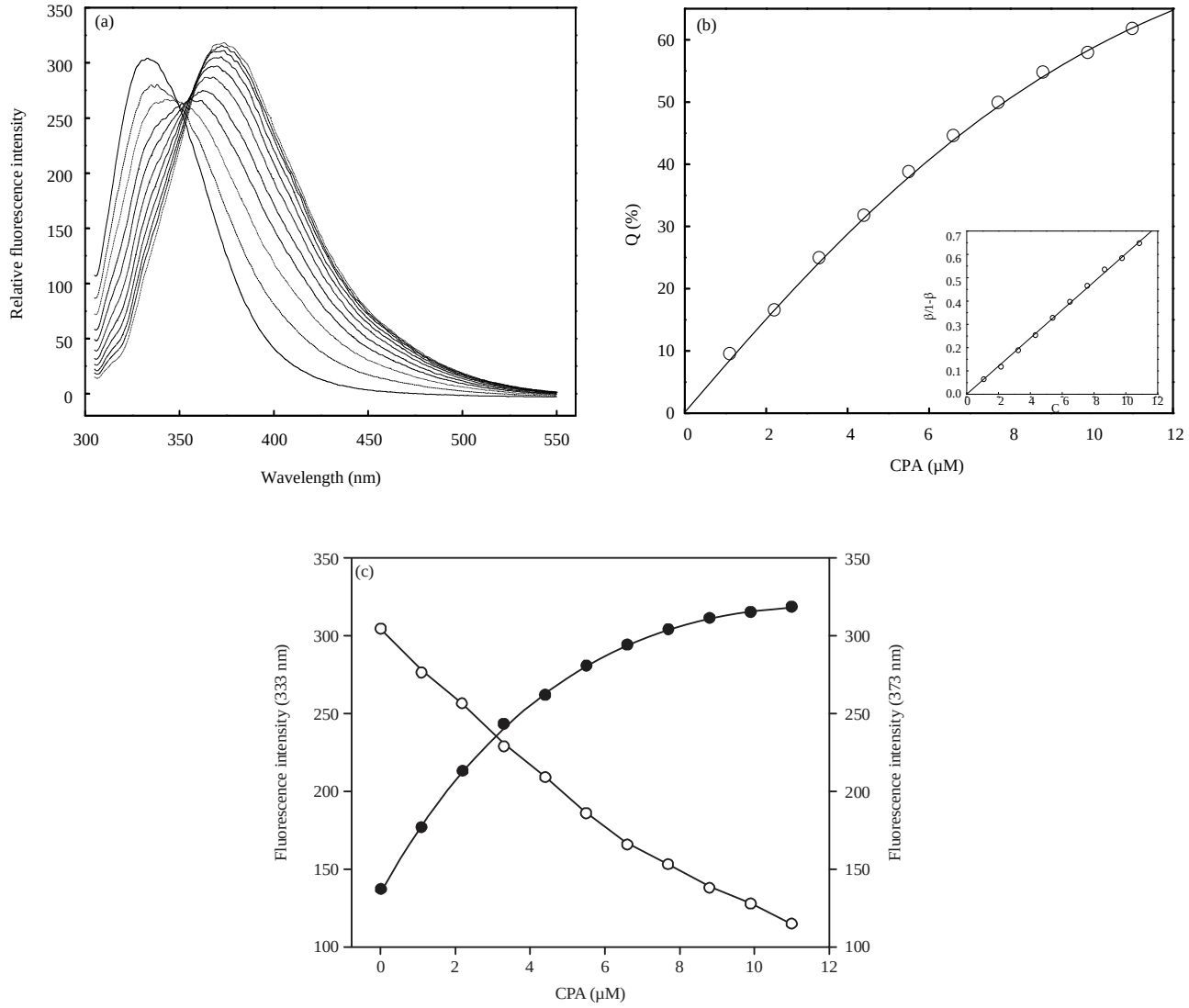

Fig. 5(a-c): (a) Quenching of intrinsic fluorescence of LOX-1 on titration with CPA, (b) Primary plot of quenching (Q) (%) versus the concentration of CPA, (Inset: Mass action plot) and (c) Concentration dependence of energy transfer between tryptophan and bound CPA

Oleate (9Z-octadecenoic acid), a C_{18} fatty acid lacks 1, 4 pentadiene system of linoleate and is reported to be a competitive inhibitor of lipoxygenase. Due to the similarity in the structure with linoleic acid, oleate binds to the linoleate binding site. The Ki value for oleate inhibited reaction was 22 ± 2 μM[26]. Effect of two synthetic fatty acids-9Z-octadecenyl sulfate (OS) and 9Z-palmitoleyl sulfate (PS) were studied on the soy LOX-1 by Ruddat *et al.*[27]. The OS inhibited LOX-1 and 15-human lipoxygenase by allosteric mechanism[27]. The OS is similar in size to linoleic acid, the natural substrate of soy LOX-1, however linoleic acid binds to catalytic site, while, OS binds to allosteric site. PS acts as competitive inhibitor versus the product 13-hydroperoxy-9,11-(Z,E)-octadecadienoic acid, with a Ki of 17.5 ± 3.8 μM. Ruddat *et al.*[27] stated that the presence of an allosteric site was important as it may open the possibility to design new class of lipoxygenase inhibitors. Anacardic acid [$C_{15:1}$; 6(8′(Z)-Pentadecenyl)salicylic acid], is a competitive inhibitor of soy LOX-1 with an IC_{50} value of 6.8 μM and Ki value of 2.8 μM. The inhibition was due to the presence of alkeneyl side chain[28]. Taxusabietane and Taxamairin inhibited LOX with the IC_{50} value of 69 and 73 μM, respectively[29], but the mechanism of inhibition is not studied. Flavanoids inhibit lipoxygenase-mediated LTB4 production in human neutrophils and the catechol group interacts and

causes the inhibition of LOX[30]. Octyl protocatechuates inhibits the activity of LOX-1. Octyl protocatechuates binds competitively to LOX-1 with a K_m of 20.9 μM and inhibition is due to the alkyl side chains[31]. A natural molecule 2,4,6-trihydroxy-3-geranyl-acetophenone isolated from *Melicope ptelefolia* exerts dose-dependent inhibiton agoinst soy LOX with an IC_{50} value of 20 μM[32]. The same group synthesized the geranylated with elongated aliphatic chain form of 2,4,6-trihydroxy-3-geranyl-acetophenone and showed enhanced inhibitory activity with an IC_{50} value of 10.31 μM[33]. The authors reported that lipophilicity of alkyl side chains brings about the inhibiton, which is in agreement with the present study. Luteolin, isolated from the seeds of *Perilla frutescens*, inhibited peroxidation of linoleic acid catalysed LOX-1 non-competitively with an IC_{50} value of 5.0 μM[34]. The CPA with a low IC_{50} value could be explored as a potential candidate for drug design.

The study highlights the inhibition and molecular interaction of CPA, a structural analogue of linoleic acid with LOX. The study explores the use of different techniques to certainly affirm the mechanism of inhibition and mode of binding. Due to the structural similarity between inhibitor and substrate, the inhibitor is binding nearby to the substrate binding cleft thus making substrate unavailable to the enzyme. This is evidenced from L-B plot and substantiated through fluorescent studies, docking and energy transfer data. Lipophilic inhibitors with similarity in structure with that of substrate belongs to non-redox class of inhibitors. The present study reveals that CPA is inhibiting competitively, with the binding site being near to the substrate binding cleft. The energy transfer and docking data suggests that CPA is closely interacting with the enzyme thus preventing the binding of substrate. The hydrophobic chain of CPA binds to the active site of LOX-1 competitively thereby decreasing the activity. The data reported herein also suggests that CPA would be inhibiting the enzyme via non-redox type. The mode of inhibition is similar to that found in anacardic acid. OS binds to allosteric site, while, alkyl side chain in octyl protocatechuates binds competitively to LOX and bring about the inhibition[32]. The binding of ligand favours the conformational change in proteins. In the present study the addition of extrinsic fluorophore, result in red shift with simultaneous measurable changes in the emission maxima of the intrinsic fluorophore. This is due to the fluorescence energy transfer between the donor (tryptophan in LOX-1) and the acceptor (CPA). The change in the fluorescence intensity is used to determine the binding constant of LOX-1 with CPA. Apart from CPA being a probe in biomembrane studies, the present data adds on to the fact that CPA as a probe could be extended to study its interaction with other proteins. The data in the current study was observed *in vitro* condition, but the biological importance of CPA as an inhibitor in biological system is largely unknown. The relevance of the *in vitro* experiments in living systems should be largely considered and evaluation about toxicity of CPA is required.

CONCLUSION

The study provides strong evidence that CPA inhibits the activity of LOX-1 by interacting at the substrate binding site. The decrease in the activity of LOX-1 is due to competitive binding of CPA to the substrate binding site, suggesting a key role of conjugated double bonds in bringing about inhibition. The docking and energy transfer studies confirm the nature of interaction of CPA with LOX-1. Considering the IC_{50} with other reported inhibitors, there is scope for the structural modification of CPA which could enhance its inhibitory activity. The study provides a promising approach to explore the potential of CPA as a candidate of drug against the LOX enzyme.

SIGNIFICANCE STATEMENT

Lipoxygenase-1 is an important enzyme activated during the inflammatory pathway. Due to the similarity in structure of CPA and linoleic acid, CPA inhibits LOX-1 by competitive mechanism. Nevertheless, with studies in the modification in structure of CPA and catalytic mechanism, the study provides a promising approach to explore the potential of CPA as a candidate of drug against the LOX enzyme by subjecting to structural modification.

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