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Design, synthesis, and biological evaluation of prenylated chalcones as 5-LOX inhibitors

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ABSTRACT

Ten novel mono- and di-*O*-prenylated chalcone derivatives were designed on the basis of a homology derived molecular model of 5-lipoxygenase (5-LOX). The compounds were docked into 5-LOX active site and the binding characteristics were quantified using LUDI. To verify our theoretical assumption, the molecules were synthesized and tested for their 5-LOX inhibitory activities. The synthesis was carried out by Claisen–Schmidt condensation reaction of mono- and di-*O*-prenylated acetophenones with appropriate aldehydes. 5-LOX in vitro inhibition assay showed higher potency of di-*O*-prenylated chalcones than their mono-*O*-prenylated chalcone analogs. Compound **5e** exhibited good inhibition with an IC_{50} at 4 μM. The overall trend for the binding energies calculated and LUDI score was in good qualitative agreement with the experimental data. Further, the compound **5e** showed potent anti-proliferative effects (GI_{50} at 9 μM) on breast cancer cell line, MCF-7.

1. Introduction

Chalcones or 1,3-diaryl-2-propene-1-ones, belong to the flavonoid family. Chemically they consist of open chain flavonoids in which the two aromatic rings are joined by a three-carbon α,β-unsaturated carbonyl system. There is currently a great deal of interest in the health benefits of phytochemicals, in particular prenylated flavonoids, because of their interesting biological activities. As revealed by structure–activity relationship (SAR) studies, the presence of isoprenoid chains of varying length and type is a major determinant of the bioactivity of prenylated flavonoids. The remarkable properties of these compounds are thought to reside in their enhanced interaction with biological membranes and increased affinity for target proteins compared with their non-prenylated analogs. Several prenylated flavonoids became the focus of attention as a result of growing interest in isoprenoid chemistry, of these, prenylated chalcones are an abundant subclass of flavonoids that are widely distributed in nature.[1–4] They are associated with a wide range of biological activities, such as anti-bacterial,[5] anti-malarial,[6] anti-fungal,[7] anti-diabetic,[8] anti-tumor,[9–11] anti-oxidative,[12] anti-inflammatory,[13] and NF-κB inhibitory activity.[14] Prenylated chalcones and their cyclic analogs were found to possess moderate to good antimicrobial, antiviral, anti-invasive, and anti-insecticidal activities.[15] Among these actions, the anti-inflammatory activity of *O*-prenylated chalcones may be mediated by the inhibition of the arachidonic acid (AA)-metabolizing enzyme, LOX.

LOXs (linoleate: oxygen oxido reductase, EC 1.13.11.12) are a group of closely related non-heme iron containing dioxygenases. These enzymes catalyze the addition of molecular oxygen into poly-unsaturated fatty acids (PUFAs) containing cis, *cis*-1,4 pentadiene structures to give their hydroperoxy derivatives. LOXs are further classified into 5-, 8-, 9-, 11-, 12-, and 15-LOXs according to the positional specificity of arachidonate oxygenation.[16] One of the LOX pathways of AA metabolism, the 5-LOX pathway is the source of potent pro-inflammatory mediators.[17] The 5-LOX acts preferentially upon unesterified AA, inserting molecular oxygen at the fifth carbon and forming the hydroperoxy intermediate, 5-hydroperoxyeicosatetraenoic acid (5-HPETE).[18] The same enzyme then catalyzes a dehydration reaction, forming the unstable epoxide intermediate, leukotriene A_4 (LTA_4). LOX metabolites are potent physiological effectors in a variety of cellular responses, associated with normal host defense and inflammation. Products of the 5-LOX pathway are thus important mediators of inflammation. Inhibitors of the 5-LOX pathway, therefore, have a therapeutic potential in a variety of inflammatory and allergic diseases. These efforts have resulted in the release of Zileuton (5-LOX inhibitor) and Montelukast [LT receptor antagonist] into the market for the treatment of asthma. 5-LOX is also associated with Gastro Esophageal Reflux Disease (GERD). Elevated levels of LTB_4 have been found in blood and joint fluid from patients with rheumatoid arthritis[19] and in

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colonic mucosa from patients with ulcerative colitis or Crohn's disease. LOX and their products are shown to play an important role in tumor formation and cancer metastasis.[20] High expression of 5-LOX was found in prostate, lung, colon, breast, and other cancer cell lines.[21–23] Recently it has been shown that 5-LOX (ALOX5) is critical regulator for leukemia cancer stem cells (LSCS) in chronic myeloid leukemia (CML). Treatment of CML mice with a 5-LOX inhibitor also impaired the function of LSCS.[24] Inhibition of the 5-LOX pathway may also be useful for cancer therapy. 5-LOX inhibitor has been shown to prevent lung tumorigenesis in carcinogen-treated mice.[25] As 5-LOX has been shown to have a major role in the pathogenesis of various inflammatory disorders, including cancer, inhibitors of 5-LOX will have wide ranging therapeutic applications.[23]

Prenylated flavonoids have become the focus of attention as a result of growing interest in isoprenoid chemistry. Thus, in this study, prenylated chalcones were designed, synthesized, and tested for their 5-LOX inhibition activity. After evaluating the compounds for inhibition of 5-LOX in vitro, the compounds were further tested for their anti-proliferative effects on breast (MCF-7) and colon (COLO-205) cancer cell lines. These cell lines were selected as 5-LOX inhibitors are known to exhibit anti-proliferative effects in these cell lines by inducing apoptosis.[26,27] Further, to check the toxicity of the best compound, it was tested on human keratinocyte cell line, HaCaT.

2. Results and discussion

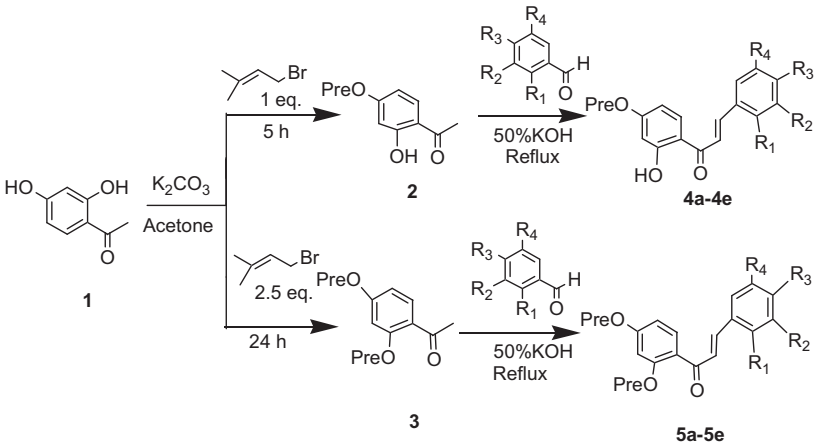
2.1. Synthesis

Prenylated chalcones (**4a–e** and **5a–e**) were synthesized by a base catalyzed Claisen–Schmidt condensation of appropriately substituted prenylated acetophenone with substituted benzaldehyde in EtOH (Scheme 1). The prenylated acetophenones **2**[28] and **3**[29] were prepared from 2,4-dihydroxy acetophenone with prenyl bromide and anhydrous K_2CO_3 in acetone. This method for the preparation of prenylated chalcones is attractive, as observed from ^{1}H NMR spectra, the coupling constants of the two olefinic protons were around 16 Hz (*trans*-configuration). There is no need for protection of hydroxyl groups during this procedure and it also resulted in good yield. The structures of all the 10 prenylated chalcones were established on the basis of IR, ^{1}H NMR, ^{13}C NMR, and LCMS spectral data.

2.2. Docking and scoring

The structures of the ligands were energy minimized to a RMSD of 0.001 kcal/mol using MMFF94X forcefield. The ligands (prenylated chalcones and NDGA) were docked into 5-LOX active site and the best and the most energetically favorable conformation of each ligand were selected. A four-stage protocol was set up for energy minimizations of the protein–inhibitor complex using AMBER. Minimization methods were used for predicting relative binding affinities of the inhibitors using energies obtained both in solvent as well as complex phases of each inhibitor. The binding affinities of the prenylated chalcones in the optimized complexes was further estimated using LUDI. The di-*O*-prenylated chalcones were predicted to be more effective than their mono-*O*-prenylated chalcones analogs (Table 1). The high affinity of compound **5e** (Fig. 1), as calculated in the *Ludi* module and by minimization methods indicates that it can form a potential 5-LOX inhibitor candidate.

A comparison of SAR data show that the LUDI score and binding energies increase as the number of hydrogen bonds increase. In the case of the most highly scored compound **5e** most of the functional groups interacted favorably with the 5-LOX active site. Strong hydrogen bond interactions were observed between 3-methoxy of compound **5e** and Thr784 (O···HO, 3.7 Å), 4-methoxy and His271 (O···HN, 2.4 Å), and 5-methoxy and Asp560 (O···HO, 2.3 Å), respectively. The 2′-hydroxyl is replaced with 2′-*O*-prenyl group in compound **5e** in comparison to compound **4e**. In mono-*O*-prenyl compounds, it has been observed that the 2′-hydroxyl group formed hydrogen bond interactions with Ser567 (O···HO, 2.5 Å). The 4′-*O*-prenyl group in compound **4a–e** formed strong hydrophobic interactions with Trp526 (3.4 Å) and Leu572 (4 Å). In compound **5a–e**, it has been observed that the prenyl group of



4a, 5a $R_1 = R_2 = R_4 = H$, $R_3 = OMe$

4b, 5b $R_1 = R_2 = OMe$, $R_3 = R_4 = H$

4c, 5c $R_1 = R_2 = R_3 = OMe$, $R_4 = H$

4d, 5d $R_1 = R_4 = H$, $R_2 = R_3 = OMe$

4e, 5e $R_1 = H$, $R_2 = R_3 = R_4 = OMe$

Scheme 1. Route for the synthesis of prenylated chalcones (pre denotes prenyl group).

Table 1
Binding energies, LUDI scores, and in vitro 5-LOX enzyme inhibition assay data for the prenylated chalcones

Compound	IC_{50} (μM)	LUDI score	ΔE_{bind} (total) (kcal/mol)
4a	>100	752	−14.4
4b	>100	613	−14.5
4c	>100	690	−15.1
4d	>100	726	−16.6
4e	>100	768	−17.5
5a	>100	710	−21.2
5b	22	766	−21.6
5c	49	779	−22.9
5d	9	785	−24.4
5e	4	875	−25.1
NDGA	1.5	882	−25.5

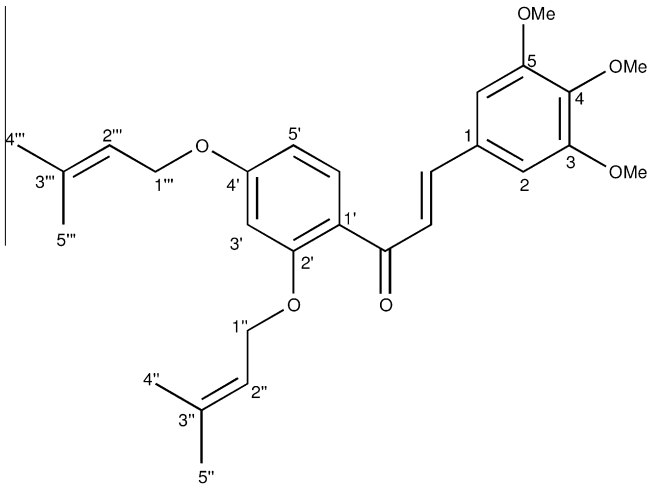


Figure 1. Structure of compound **5e**.

the 2′-*O*-prenyl group formed strong hydrophobic interactions with Leu255 (3.1 Å) and Lys283 (3.4 Å) along with strong hydrogen bond interactions between oxygen atom of 2′-*O*-prenyl and Ser567 (Fig. 2). The interaction analysis of the compounds suggests that compound **5e** is the most potential molecule.

NDGA is firmly bound in the open cavity, that is, in the sixth coordination of the iron atom as reported in our earlier studies.[31] NDGA has four hydroxyl groups, the first hydroxyl group formed strong hydrogen bonding interactions with the side chain of His271 (O···Nε, 2.75 Å), the second hydroxyl group formed hydrogen-bonding interactions with the main chain of Asp276 (OH···O, 2.4 Å), the third hydroxyl group formed hydrogen bonding with the side chain atom of Ile564 (OH···O, 2.86 Å) and two bonds with Asn565 (O···N, 3.16 Å). The fourth hydroxyl group is in close proximity to the His530 (O···N, 3.9 Å). Hence it exhibits very low IC_{50} and possesses high affinity. The binding energies calculated and the LUDI scores estimated for the compound 5e indicate that it may be as potential as NDGA. To verify our theoretical findings the molecules were further synthesized and tested for their 5-LOX inhibitory activities.

2.3. In vitro 5-LOX enzyme assay

The potential 5-LOX inhibitory molecules synthesized were tested in vitro for their inhibitory properties against 5-LOX enzyme using the assay described by Reddanna et al.[30] Out of the 10 molecules tested compounds **5d** and **5e** showed inhibition at a concentration <10 μM. Compounds **5b** and **5c** showed IC_{50} <50 μM. All the mono-*O*-prenyl chalcones, on the other hand, showed IC_{50} >100 μM. The most active molecule, compound **5e**, showed potent inhibition of 5-LOX with an IC_{50} value of 4 μM (Table 1). The binding energies estimated were in good qualitative agreement with the experimental data than the LUDI scores calculated. As estimated the di-*O*-prenylated chalcones were more potent than the mono-*O*-prenyl chalcones. The number of hydrogen bonds in the molecules showed inverse correlation with the experimental IC_{50}. Further, the inhibitory activity of the best inhibitor, compound **5e**, was analyzed and confirmed by analysis of products on straight phase-HPLC (SP-HPLC). It has been observed in HPLC studies that the compound **5e** at 4 μM effectively inhibited the production of 5-HPETE from 5-LOX as shown in Figure 3.

2.4. In vitro anti-proliferative effects on cancer cell lines

Prenylated chalcones were further tested for their anti-proliferative effects on MCF-7 and COLO-205 cell lines. Cells were incubated with different concentrations of compounds for 24 h and the cell viability was measured by MTT assay. The GI_{50} values for different compounds obtained are shown in Table 2 and Figure 4. The compounds showed anti-proliferative effects in a concentration dependent manner. The di-*O*-prenyl compounds (**5a–e**) showed better anti-proliferative effects than their mono-*O*-prenyl derivatives (**4a–e**). The toxicity of the potential compound 5e when tested on normal cell line (HaCaT), showed no appreciable effects up to 100 μM.

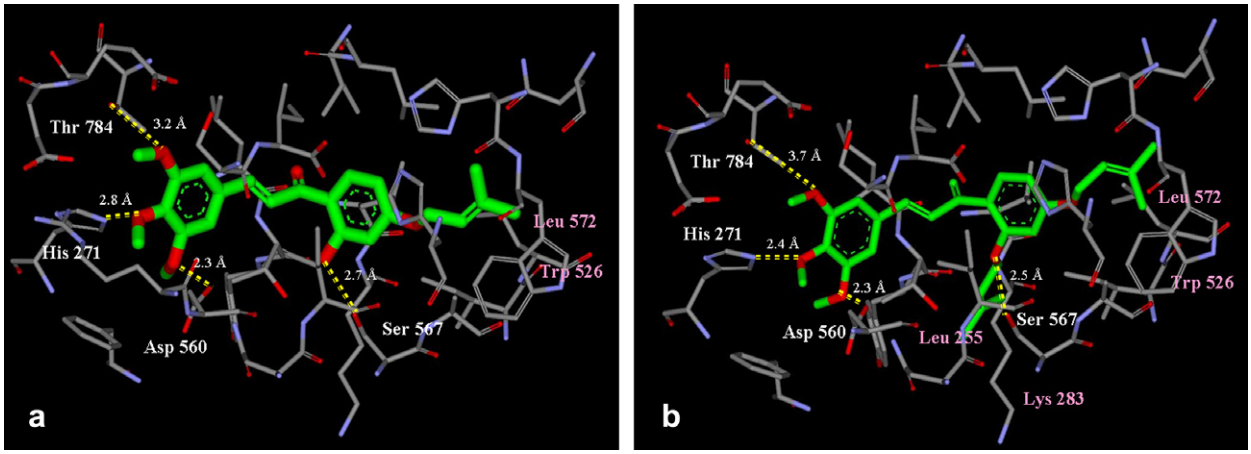


Figure 2. Hydrogen bonding of the compounds with 5-LOX active site residues: (a) compound **4e** and (b) compound **5e** (hydrogen bonding interactions are shown as dotted line, hydrogen bond forming amino acids in active site are labeled in white and hydrophobic interactions forming amino acids are labeled in pink).

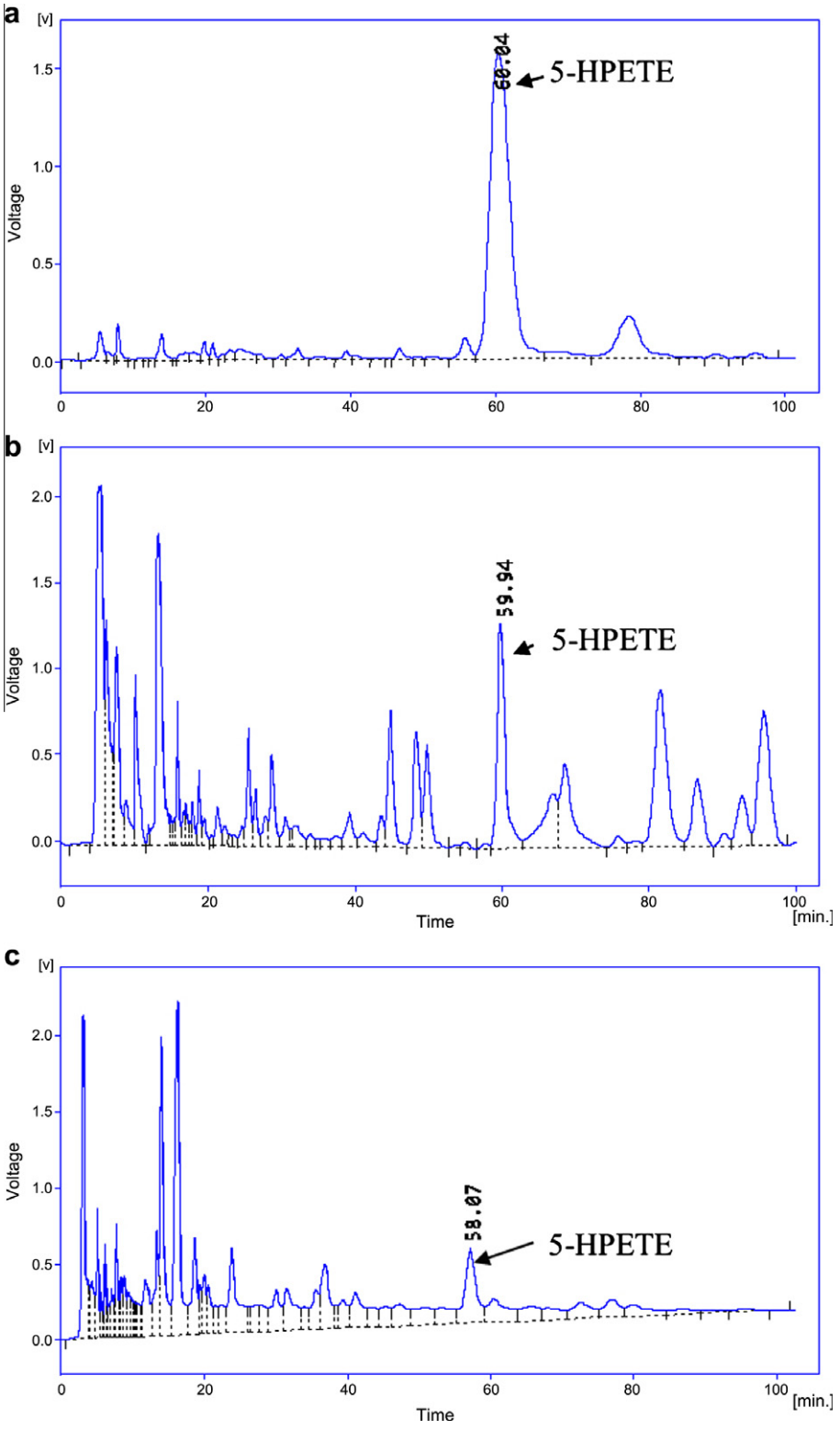


Figure 3. HPLC product profile of 5-HPETE generated from the reaction of potato 5-LOX with AA as the substrate. (a) Standard 5-HPETE. (b) 5-LOX products in the control. (c) 5-LOX products in the presence of compound **5e** (4 μM). HPLC conditions were as described under experimental procedures. UV absorbance of the eluate was monitored at 235 nm.

3. Conclusions

A new class of prenylated chalcones has been designed and synthesized by Claisen–Schmidt condensation reaction and evaluated for their 5-LOX inhibitory and anti-proliferative effects. The overall trend for the binding energies estimated is in good qualitative agreement with the experimental data than the LUDI scores. In addition, all of the lead molecules were designed on the basis of a homology derived molecular model of 5-LOX. Compound **5e** inhibited 5-LOX with an IC_{50} of 4 μM. Further it showed potent

Table 2
Cytotoxicity of prenylated chalcone derivatives against human cancer cell lines, MCF-7 and COLO cells

Compound	GI_{50} (μM)	
	MCF-7	COLO
4a	97.5	>100
4b	>100	>100
4c	>100	>100
4d	>100	>100
4e	81	96
5a	67.5	97
5b	73	99
5c	92	>100
5d	66.5	90
5e	9	78

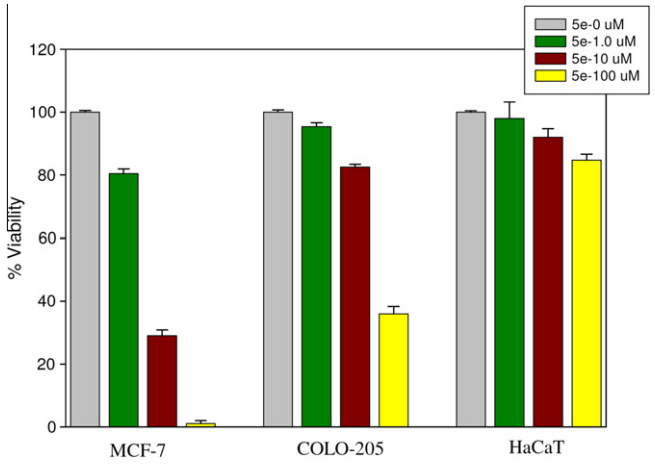


Figure 4. MCF-7, COLO-205, and HaCaT cells were cultured for 24 h with or without **5e** (1–100 μM) and cell viability was analyzed by MTT assay. Each data point represents the mean of four independent experiments with error bars indicating ±SE.

anti-proliferative effects on breast cancer cell line, MCF-7. These novel inhibitors developed could form leads for further inhibitor development against 5-LOX. This study assumes importance in the light of key role played by 5-LOX in various pathological manifestations.

4. Experimental section

4.1. Chemistry

4.1.1. General information

Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. The reactions were monitored by thin layer chromatography (TLC) on precoated Merck Silica gel $60F_{254}$ aluminum plates. IR spectra were recorded in KBr disks on a JASCO FT/IR-5300. ^{1}H NMR spectra were recorded on Bruker Avance 400 spectrometer using $CDCl_3$ with TMS as internal standard. Coupling constant (J) values are calculated in hertz (Hz) and spin multiples are given as s (singlet), d (double), t (triplet), q (quartet), m (multiplet), and br (broad). Mass spectra were recorded on either VG7070H mass spectrometer using EI technique or Shimadzu-LCMS-2010A. Column chromatography was performed on silica gel (Merk) 100–200 mesh.

4.1.2. Synthesis of 2-hydroxy–4-*O*-prenylacetophenone (2)

To a solution of 2,4-dihydroxyacetophenone (**1**) (5.32 g, 1 equiv, 35 mmol) in acetone (100 mL) was added freshly ignited K_2CO_3 (3 g) and prenyl bromide (4.07 mL, 1 equiv, 35 mmol). The reaction mixture was refluxed for 5 h. On completion of the reaction (TLC), solvent was evaporated under reduced pressure and ice cold water (75 mL) was added to it, and extracted with ethyl acetate (3 × 100 mL). The combined organic layer was separated, dried over Na_2SO_4, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography eluting with hexane/EtOAc (8:2) to afford the title compound **2** as white crystalline solid (5.42 g) in 70.3% yield.

^{1}H NMR (400 MHz, $CDCl_3$): δ 12.76 (1H, s, OH-2), 7.62 (1H, d, J = 7.6 Hz, H-6), 6.48–6.43 (2H, m, H-5, H-3), 5.51–5.46 (1H, m, CH-2′), 4.56 (2H, d, J = 6.8 Hz, CH_2-1′), 2.57 (3H, s, $COCH_3$), 1.82 (3H, s, prenyl-CH_3), 1.79 (3H, s, prenyl-CH_3). LCMS (positive mode) m/z 221 $[M+H]^+$.

4.1.3. Synthesis of 2,4-di-*O*-prenylacetophenone (3)

To a solution of 2,4-dihydroxyacetophenone (**1**) (5.32 g, 1 equiv, 35 mmol) in acetone (100 mL) was added freshly ignited K_2CO_3 (10 g) and prenyl bromide (10.18 mL, 2.5 equiv, 87.5 mmol). The reaction mixture was refluxed for 24 h. On completion of the reaction (TLC), cooling to room temperature, filtration, and the residue was purified by silica gel column chromatography eluting with hexane/EtOAc (7:3) to afford the title compound **3** as pale yellow solid (8.12 g) in 80.5% yield.

^{1}H NMR (400 MHz, $CDCl_3$): δ 7.82 (1H, d, J = 8.4 Hz, H-6), 6.50 (1H, dd, J = 8.4, 2.0 Hz, H-5), 6.47 (1H, d, J = 2.0 Hz, H-3), 5.50 (2H, br s, CH-2′, CH-2″), 4.58–4.54 (4H, m, CH_2-1′, CH_2-1″), 2.58 (3H, s, $COCH_3$), 1.80 (3H, s, prenyl-CH_3), 1.75 (3H, s, prenyl-CH_3), 1.74 (3H, s, prenyl-CH_3), 1.70 (3H, s, prenyl-CH_3). LCMS (positive mode) m/z 289 $[M+H]^+$.

4.1.4. General procedure for the synthesis of prenylated chalcones

Prenylated chalcones (**4a–e** and **5a–e**) were synthesized by a base catalyzed Claisen–Schmidt condensation reaction of mono- and di-*O*-prenylated acetophenone (1.0 equiv, 1 mmol) and a substituted benzaldehyde (1.0 equiv, 1.0 mmol). The starting materials dissolved in a minimum volume of ethanol (10–15 mL) and added aqueous solution of 50% KOH (5 equiv). The reaction mixture was refluxed for 3–4 h. Until the completion of reaction by TLC, the reaction mixture was concentrated to one-fourth of its original volume and pH was adjusted to 3–4 with 2 N HCl solution and then extracted with ethyl acetate (3×100 mL). The combined organic layer was separated, dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The residue was purified by column chromatography over silica gel, eluting with step gradient of hexane/EtOAc to afford pure prenylated chalcone derivatives **4a–e** and **5a–e**.

4.1.4.1. 2′–Hydroxy-4-methoxy-4′-*O*-prenylchalcone (4a). Orange color semi-solid (0.27 g); yield 79.8%; IR (KBr) ν_{max}: 3350, 2930, 2858, 1633, 1568, 1367, 1253, 1032 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 13.41 (1H, s, OH-2′), 7.86 (1H, d, J = 16.0 Hz, H-β), 7.62 (1H, d, J = 8.7 Hz, H-6′), 7.48 (1H, d, J = 16.0 Hz, H-α), 7.31 (2H, d, J = 8.0, H-2, H-6), 6.96 (2H, d, J = 8.0 Hz, H-3, H-5), 6.50–6.46 (2H, m, H-5′, H-3′), 5.49 (1H, t, J = 6.6 Hz, H-2″), 4.56 (2H, d, J = 6.6 Hz, CH_2-1″), 3.87 (3H, s, OMe), 1.81 (3H, s, prenyl-CH_3), 1.76 (3H, s, prenyl-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 191.85 (C=O), 166.58 (C-2′), 165.22 (C-4′), 161.80 (C-4), 144.20 (C-β), 139.15 (C-3″), 132.26 (C-6′), 130.36 (C-2 and C-6), 128.66 (C-1), 118.74 (C-2″), 117.90 (C-α), 114.48 (C-3 and C-5), 113.98 (C-1′), 108.20 (C-5′), 101.72 (C-3′), 65.18 (C-1″), 55.44 (OMe), 25.82 (Me-4″), 18.25 (Me-5″). Anal. Calcd for $C_{21}H_{22}O_4$: C, 74.54; H, 6.55. Found: C, 74.61; H, 6.51. LCMS (positive mode) m/z 339 $[M+H]^+$.

4.1.4.2. 2′–Hydroxy-2,3-dimethoxy-4′-*O*-prenylchalcone (4b). Yellow solid (0.305 g); yield 82.8%; mp 95–96 °C; IR (KBr) ν_{max}: 3439, 2930, 2843, 1641, 1575, 1359, 1269, 1072 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 13.49 (1H, s, OH-2′), 8.18 (1H, d, J = 15.6 Hz, H-β), 7.84 (1H, d, J = 9.0 Hz, H-6′), 7.68 (1H, d, J = 15.6 Hz, H-α), 7.30 (1H, dd, J = 9.0, 2.0 Hz, H-6), 7.16 (1H, dd, J = 9.0, 9.0 Hz, H-5), 7.00 (1H, dd, J = 9.0, 2.0 Hz, H-4), 6.52–6.49 (2H, m, H-5′, H-3′), 5.51 (1H, t, J = 6.7 Hz, H-2″), 4.58 (2H, d, J = 6.7 Hz, CH_2-1″), 3.91 (3H, s, OMe), 3.89 (3H, s, OMe), 1.82 (3H, s, prenyl-CH_3), 1.77 (3H, s, prenyl-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 192.10 (C=O), 166.58 (C-2′), 165.48 (C-4′), 153.22 (C-3), 148.98 (C-2), 139.18 (C-β and C-3″), 131.21 (C-6′), 128.98 (C-α), 124.17 (C-5), 121.86 (C-1), 119.74 (C-2″), 118.65 (C-6), 114.27 (C-4), 114.06 (C-1′), 108.20 (C-5′), 101.68 (C-3′), 65.15 (C-1″), 61.31 (OMe), 55.88 (OMe), 25.78 (Me-4″), 18.21 (Me-5″). Anal. Calcd for $C_{22}H_{24}O_5$: C, 71.72; H, 6.57. Found: C, 71.65; H, 6.52. LCMS (positive mode) m/z 369 $[M+H]^+$.

4.1.4.3. 2′–Hydroxy-2,3,4-trimethoxy-4′-*O*-prenylchalcone (4c). Light yellowish oil (0.315 g); yield 79.1%; IR (KBr) ν_{max}: 3445, 2932, 2849, 1631, 1494, 1359, 1257, 1097 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 13.60 (1H, s, OH-2′), 8.05 (1H, d, J = 15.6 Hz, H-β), 7.81 (1H, d, J = 8.8 Hz, H-6′), 7.63 (1H, d, J = 15.6 Hz, H-α), 7.38 (1H, d, J = 8.8 Hz, H-6), 6.72 (1H, d, J = 8.8 Hz, H-5), 6.49–6.47 (2H, m, H-5′, H-3′), 5.48 (1H, br s, H-2″), 4.56 (2H, d, J = 6.4 Hz, CH_2-1″), 3.97 (3H, s, OMe), 3.91 (3H, s, OMe), 3.89 (3H, s, OMe), 1.81 (3H, s, prenyl-CH_3), 1.75 (3H, s, prenyl-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 192.19 (C=O), 166.57 (C-2′), 165.33 (C-4′), 155.95 (C-4), 153.90 (C-2), 142.49 (C-3), 139.73 and 139.02 (C-β and C-3″), 131.12 (C-6′), 128.83 (C-α), 124.20 (C-6), 119.45 (C-2″), 118.78 (C-1′), 114.15 (C-1), 108.08 (C-5′), 107.80 (C-5), 101.71 (C-3′), 65.15 (C-1″), 61.38 (OMe), 60.90 (OMe), 56.17 (OMe), 25.80 (Me-4″), 18.22 (Me-5″). Anal. Calcd for $C_{23}H_{26}O_6$: C, 69.33; H, 6.58. Found: C, 69.21; H, 6.62. LCMS (positive mode) m/z 399 $[M+H]^+$.

4.1.4.4. 2′–Hydroxy-3,4-dimethoxy-4′-*O*-prenylchalcone (4d). Orange color semi-solid (0.29 g); yield 78.8%; IR (KBr) ν_{max}: 3410, 2930, 2847, 1633, 1514, 1367, 1257, 1024 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 13.40 (1H, s, OH-2′), 7.87–7.83 (2H, m, H-β, H-6′), 7.44 (1H, d, J = 15.6 Hz, H-α), 7.25 (1H, d, J = 2.1 Hz, H-2), 7.17 (1H, dd, J = 8.4, 2.1 Hz, H-6), 6.90 (1H, d, J = 8.4, Hz, H-5), 6.51–6.49 (2H, m, H-5′, H-3′), 5.48 (1H, br s, H-2″), 4.56 (2H, d, J = 6.5 Hz, CH_2-1″), 3.97 (3H, s, OMe), 3.94 (3H, s, OMe), 1.81 (3H, s, prenyl-CH_3), 1.76 (3H, s, prenyl-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 191.71 (C=O), 166.59 (C-2′), 165.42 (C-4′), 151.57 (C-4), 149.28 (C-3), 144.76 (C-β), 139.13 (C-3″), 131.09 (C-6′), 128.84 (C-1), 123.34 (C-6), 118.71 (C-2″) 118.04 (C-α), 114.02 (C-1′), 111.16 (C-5), 110.22 (C-2), 108.20 (C-5′), 101.69 (C-3′), 65.19 (C-1″), 55.99 (OMe), 55.88 (OMe), 25.81 (Me-4″), 18.25 (Me-5″). Anal. Calcd for $C_{22}H_{24}O_5$: C, 71.72; H, 6.57. Found: C, 71.58; H, 6.45. LCMS (positive mode) m/z 369 $[M+H]^+$.

4.1.4.5. 2′–Hydroxy-3,4,5-trimethoxy-4′-*O*-prenylchalcone (4e). Yellow amorphous powder (0.31 g); yield 77.8%; mp 113–115 °C; IR (KBr) ν_{max}: 3440, 2939, 2841, 1639, 1572, 1371, 1271, 1124 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 13.51 (1H, s, OH-2′), 7.84–7.79 (2H, m, H-β, H-6′), 7.46 (1H, d, J = 15.6 Hz, H-α), 6.88 (2H, s, H-6, H-2), 6.49 (2H, m, H-5′, H-3′), 5.50 (1H, br s, H-2″), 4.58 (2H, d, J = 6.8 Hz, CH_2-1″), 3.94 (6H, s, 2 × OMe), 3.91 (3H, s, OMe), 1.82 (3H, s, prenyl-CH_3), 1.77 (3H, s, prenyl-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 191.58 (C=O), 166.68 (C-2′), 165.57 (C-4′), 153.51 (C-3 and C-5), 144.48 (C-β), 140.59 (C-4), 139.27 (C-3″), 131.14 (C-6′), 130.31 (C-1), 119.53 (C-2″), 118.64 (C-α), 113.07 (C-1′), 108.35 (C-5′), 105.80 (C-2 and C-6), 101.69 (C-3′), 65.23 (C-1″), 61.03 (OMe), 56.27 (2 × OMe), 25.84 (Me-4″), 18.26 (Me-5″). Anal. Calcd for $C_{23}H_{26}O_6$: C, 69.33; H, 6.58. Found: C, 69.25; H, 6.52. LCMS (positive mode) m/z 399 $[M+H]^+$.

4.1.4.6. 4-Methoxy-2′,4′-di-*O*-prenylchalcone (5a). Yellowish oil (0.305 g); yield 75.1%; IR (KBr) ν_{max}: 2930, 2849, 1687, 1575, 1425, 1255, 1020 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 7.85 (1H, d, J = 15.6 Hz, H-β), 7.62 (1H, d, J = 8.5 Hz, H-6′), 7.52 (1H, d, J = 15.8 Hz, H-α), 7.42 (2H, d, J = 8.2 Hz, H-2, H-6), 6.95 (2H, d, J = 8.4 Hz, H-3, H-5), 6.51–6.48 (2H, m, H-5′, H-3′), 5.49 (2H, t, J = 6.7 Hz, H-2″, H-2‴), 4.57 (4H, d, J = 6.7 Hz, CH_2-1″, CH_2-1‴), 3.89 (3H, s, OMe), 1.81 (3H, s, prenyl-CH_3), 1.79 (3H, s, prenyl-CH_3), 1.77 (3H, s, prenyl-CH_3), 1.69 (3H, s, prenyl-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 190.05 (C=O), 163.44 (C-2′), 161.07 (C-4′), 159.96 (C-4), 141.37 (C-β), 138.87 and 138.70 (C-3″ and C-3‴), 132.00 (C-6′), 130.36 (C-2 and C-6), 128.51 (C-1), 119.21 and 119.09 (C-2″ and C-2‴), 118.58 (C-α), 114.32 (C-3 and C-5), 113.63 (C-1′), 106.07 (C-5′), 100.38 (C-3′), 65.54 and 65.04 (C-1″ and 1‴), 55.36 (OMe), 25.84 and 25.76 (Me-4″ and Me-4‴), 18.27 and 18.24 (Me-5″ and Me-5‴). Anal. Calcd for $C_{26}H_{30}O_4$: C, 76.82; H, 7.44. Found: C, 76.71; H,7.52. LCMS (negative mode) m/z 405 $[M-H]^-$.

4.1.4.7. 2,3-Dimethoxy-2′,4′-di-*O*-prenylchalcone (5b). Yellow gummy oil (0.341 g); yield 78.2%; IR (KBr) ν_{max}: 2974, 2930, 1645, 1601, 1469, 1267, 1012 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 8.10 (1H, d, J = 15.5 Hz, H-β), 7.80 (1H, d, J = 9.0 Hz, H-6′), 7.62 (1H, d, J = 15.6 Hz, H-α), 7.25 (1H, dd, J = 8.8, 2.2 Hz, H-6), 7.06 (1H, dd, J = 8.8, 8.8 Hz, H-5), 6.91 (1H, dd, J = 8.8, 2.2 Hz, H-4), 6.58–6.45 (2H, m, H-5′, H-3′), 5.52 (2H, t, J = 6.7 Hz, H-2″, H-2‴), 4.58 (4H, d, J = 6.7 Hz, CH_2-1″, CH_2-1‴), 3.88 (3H, s, OMe), 3.86 (3H, s, OMe), 1.81 (3H, s, prenyl-CH_3), 1.77 (3H, s, prenyl-CH_3), 1.72 (3H, s, prenyl-CH_3), 1.62 (3H, s, prenyl-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 190.22 (C=O), 163.58 (C-2′), 160.07 (C-4′), 153.19 (C-3), 148.81 (C-2), 139.10 (C-β), 138.87 and 138.64 (C-3″ and 3‴), 130.95 (C-6′), 128.88 (C-α), 124.23 (C-5), 121.89 (C-1), 119.79 and 119.20 (C-2″ and C-2‴), 118.72 (C-6), 114.28 (C-4), 113.48 (C-1′), 106.12 (C-5′), 100.31 (C-3′), 65.55 and 65.05 (C-1″ and C-1‴), 61.42 (OMe), 55.87 (OMe), 25.83 and 25.73 (Me-4″ and Me-4‴), 18.24 (Me-5″ and Me-5‴). Anal. Calcd for $C_{27}H_{32}O_5$: C, 74.29; H, 7.39. Found: C, 74.21; H, 7.33. LCMS (positive mode) m/z 437 $[M+H]^+$.

4.1.4.8. 2,3,4-Trimethoxy-2′,4′-di-*O*-prenylchalcone (5c). Light yellowish oil (0.361 g); yield 77.4%; IR (KBr) ν_{max}: 2930, 2854, 1630, 1494, 1365, 1284, 1097 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 8.04 (1H, d, J = 15.6 Hz, H-β), 7.82 (1H, d, J = 9.2 Hz, H-6′), 7.63 (1H, d, J = 15.6 Hz, H-α), 7.39 (1H, d, J = 8.8 Hz, H-6), 6.74 (1H, d, J = 8.8 Hz, H-5), 6.50–6.43 (2H, m, H-5′, H-3′), 5.49 (2H, br s, H-2″, H-2‴), 4.57 (4H, d, J = 6.0 Hz, CH_2-1″, CH_2-1‴), 3.97 (3H, s, OMe), 3.94 (3H, s, OMe), 3.90 (3H, s, OMe), 1.81 (3H, s, prenyl-CH_3), 1.76 (3H, s, prenyl-CH_3), 1.72 (3H, s, prenyl-CH_3), 1.68 (3H, s, prenyl-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 192.18 (C=O), 166.52 (C-2′), 165.29 (C-4′), 155.90 (C-4), 153.80 (C-2), 142.55 (C-3), 139.72 (C-3″ and C-3‴), 139.04 (C-β), 131.08 (C-6′), 128.80 (C-α), 124.20 (C-6), 119.47 (C-2″ and C-2‴), 118.71 (C-1′), 114.11 (C-1), 108.08 (C-5′), 107.58 (C-5), 101.66 (C-3′), 65.12 (C-1″ and C-1‴), 61.35 (OMe), 60.88 (OMe), 56.05 (OMe), 25.77 (Me-4″ and Me-4‴), 18.19 (Me-5″ and 5‴). Anal. Calcd for $C_{28}H_{34}O_6$: C, 72.08; H, 7.35. Found: C, 72.21; H, 7.38. LCMS (positive mode) m/z 467 $[M+H]^+$.

4.1.4.9. 3,4-Dimethoxy-2′,4′-di-*O*-prenylchalcone (5d). Yellowish oil (0.338 g); yield 77.5%; IR (KBr) ν_{max}: 2928, 2866, 1645, 1514, 1375, 1261, 1020 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$): δ 7.81 (1H, d, J = 15.5 Hz, H-β), 7.78 (1H, d, J = 8.4 Hz, H-6′), 7.72 (1H, d, J = 15.5 Hz, H-α), 7.52 (1H, d, J = 2.2 Hz, H-2), 7.18 (1H, dd, J = 9.0,

2.2 Hz, H-6), 6.87 (1H, d, J = 9.0 Hz, H-5), 6.58–6.52 (2H, m, H-5′, H-3′), 5.50 (2H, br s, H-2″, H-2‴), 4.57 (4H, d, J = 6.7 Hz, CH_2-1″, CH_2-1‴), 3.97 (3H, s, OMe), 3.91 (3H, s, OMe), 1.81 (3H, s, prenyl-CH_3), 1.76 (3H, s, prenyl-CH_3), 1.74 (3H, s, prenyl-CH_3), 1.72 (3H, s, prenyl-CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 190.71 (C=O), 167.63 (C-2′), 163.35 (C-4′), 150.81 (C-4), 149.10 (C-3), 144.48 (C-β), 138.88 and 138.29 (C-3″ and C-3‴), 132.22 (C-6′), 128.83 (C-1), 122.45 (C-6), 119.20 and 119.05 (C-2″ and C-2‴) 118.68 (C-α), 114.20 (C-1′), 111.09 (C-5), 110.36 (C-2), 106.05 (C-5′), 100.51 (C-3′), 65.74 and 65.02 (C-1″ and C-1‴), 55.95 (OMe), 55.86 (OMe), 25.81 and 25.74 (Me-4″ and Me-4‴), 18.28 and 18.22 (Me-5″ and Me-5‴). Anal. Calcd for $C_{27}H_{32}O_5$: C, 74.29; H, 7.39. Found: C, 74.36; H, 7.31. LCMS (positive mode) m/z 437 $[M+H]^+$.

4.1.4.10. 3,4,5-Trimethoxy-2′,4′-di-*O*-prenylchalcone (5e). Yellowish gummy oil (0.368 g); yield 78.9%; IR (KBr) ν_{max}: 2970, 2934, 1633, 1583, 1379, 1273, 1008 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.80 (1H, d, J = 8.4 Hz, H-6′), 7.61 (1H, d, J = 16.0 Hz, H-β), 7.44 (1H, d, J = 16.0 Hz, H-α), 6.83 (2H, s, H-6, H-2), 6.59–6.44 (2H, m, H-5′, H-3′), 5.49 (2H, br s, H-2″, H-2‴), 4.58 (4H, d, J = 6.6 Hz, CH_2-1″, CH_2-1‴), 3.94 (3H, s, OMe), 3.85 (3H, s, OMe), 3.83 (3H, s, OMe), 1.82 (3H, s, prenyl-CH_3), 1.77 (3H, s, prenyl-CH_3), 1.77 (6H, s, prenyl-2×CH_3) ^{13}C NMR (100 MHz, $CDCl_3$): δ 191.60 (C=O), 166.50 (C-2′), 163.55 (C-4′), 153.53 and 153.39 (C-3 and C-5), 144.49 (C-β), 141.70 (C-4), 138.94 and 138.20 (C-3″ and C-3‴), 132.98 (C-6′), 131.20 (C-1), 119.57 and 119.16 (C-2″ and C-2‴), 118.67 (C-α), 113.10 (C-1′), 106.80 (C-5′), 105.87 (C-2 and C-6), 101.73 (C-3′), 65.89 and 65.08 (C-1″ and C-1‴), 60.98 (OMe), 56.28 (2 × OMe), 25.81 (Me-40 and Me-4‴), 18.24 (Me-5″ and Me-5‴). Anal. Calcd for $C_{28}H_{34}O_6$: C, 72.08; H, 7.35. Found: C, 72.18; H, 7.29. LCMS (negative mode) m/z 465 $[M-H]^-$.

4.2. Computational studies

As the crystal structure of 5-LOX is not yet available, the homology model of 5-LOX reported by us earlier was used in the study.[31] Ten novel mono- and di-*O*-prenylated chalcone derivatives were designed. The structure of the molecules was sketched and minimized using cerius2 and the final structure was obtained in conformational analysis. GOLD (Genetic Optimization of Ligand Docking), a docking program based on genetic algorithm[32] was used to dock the inhibitors. During docking, the default algorithm speed was selected. The number of poses for each inhibitor was set to 100, and early termination was allowed if the top three bound conformations of a ligand were within 1.5 Å RMSD.

After docking, the individual binding poses of each ligand were observed and their interactions with the protein were studied. The best and the most energetically favorable conformation of each ligand were selected and complexed with 5-LOX model. A four-stage protocol[33,34] was set up for energy minimizations of the protein–inhibitor complex using AMBER.[35] Minimization at each stage was performed using 200 steps of steepest descent and 1500 steps of conjugate gradient algorithms for minimization. In the first stage, only the solvent molecules were minimized, keeping the inhibitor and the protein (in the complex calculation) fixed. The purpose of this step is to relieve any bad contacts involving solvent molecules in the initially solvated system. In the second stage, only hydrogens in the system were allowed to relax. This step relaxes the hydrogen atoms prior to relaxing heavy atoms. It was performed because the adjustments in hydrogen atom locations are necessary to improve hydrogen bond geometries. In the third stage, all atoms of the protein were fixed, while allowing all the atoms of the inhibitor and the solvent to move during optimization. This stage allows for the relaxation of the inhibitor with respect to the protein and establishes the preferred interactions (e.g., hydrogen bonds). In the fourth and final stage, all the atoms of residues within 15 Å from the center of the inhibitor (solvent molecules, protein atoms, and the ligand) were allowed to relax.

A four-stage protocol was also established for energy minimization of the solvated inhibitor. These minimizations were carried out using periodic boundary conditions in all directions and each stage involved 100 steps of steepest descent and 1500 steps of conjugate gradient optimization. In the first stage of minimization, only the waters were minimized keeping the inhibitor (i.e., the solute) fixed. In the second stage, only hydrogens in the system were allowed to relax. In the third stage, atoms common to the ligand in the crystal structure complex and the modified ligand were also fixed, while allowing the solvent and the modified group in the ligand to move during optimization. In the fourth stage of the solvent calculation, all water molecules and the solute (ligand) were allowed to relax.

The minimized structures for all the inhibitors in the complexed and solvated states were used for calculating the following energy variables:

$$\Delta E_{bind}(\text{Intra}) = E_{com}(\text{Intra}) - E_{sol}(\text{Intra}) \quad (1)$$

$$\Delta E_{bind}(\text{Inter}) = E_{com}(\text{Inter}) - E_{sol}(\text{Inter}) \quad (2)$$

where ΔE_{bind}(intra) and ΔE_{bind}(inter) are relative intra and intermolecular binding interaction energies of a ligand, respectively, and E_{com}(Intra), E_{com}(Inter), E_{sol}(intra), and E_{sol}(Inter) are intra and intermolecular interaction energies of a ligand in the complexed and solvated states, respectively. The total binding energy, ΔE_{bind} (Total), of an inhibitor is given by

$$\Delta E_{bind}(\text{Total}) = \Delta E_{bind}(\text{Intra}) + \Delta E_{bind}(\text{Inter}) \quad (3)$$

In Table 1, the experimentally measured IC_{50}'s are compared with the binding energies calculated using minimization methods and with scores obtained using LUDI. After four-stage protocol energy minimizations, the optimized structures of protein and ligand were visualized and studied using InsightII.

The LUDI method for de novo design of ligands for proteins (i.e., enzyme inhibitors) is a method for screening a large number of compounds by analyzing the geometrical fit of given chemicals in the designated protein binding site. Other determinants of good binding are also calculated and include hydrogen bond formation, lipophilic interactions, ionic interactions, and acyclic interactions. However, LUDI can also score protein ligand interactions by statistically evaluating the fit of potential ligands. The LUDI scoring method[36] of interactions between a protein and its ligand was used in this study to quantify the binding characteristics of the compounds to 5-LOX. In general a higher LUDI score represents a higher affinity and stronger binding of a ligand to the receptor:

$$\text{LUDI score} = -73.33\,\text{mol/kcal}\ \Delta G$$

where

$$\Delta G = \Delta G_o + \Delta G_{hb} f(\Delta R) f(\Delta a) + \Delta G_{ion} f(\Delta R) f(\Delta a) + \Delta G_{lipo} A_{lipo} + \Delta G_{rot} NR\Delta G;$$

ΔG_o represents the contribution to the binding energy that does not directly depend on any specific interactions with the receptor, ΔG_{hb} and ΔG_{ion} represent the contribution from an ideal hydrogen bond and unperturbed ionic interactions, respectively, ΔG_{lipo} represents the contribution from lipophilic interactions which is proportional to the lipophilic surface A_{lipo}, $\Delta G_{rot}NR$ represents the contribution due to freezing of internal degrees of freedom in the fragment, NR the number of acylic bonds, ΔR the deviation of the hydrogen bond length from the ideal value of 1.9 Å, and Δa is the deviation of the hydrogen bond angle from the ideal value of 180°. In general, a higher LUDI score (0–1100 in range) represents higher affinity and stronger binding of a ligand to the receptor.

4.3. In vitro 5-LOX inhibitory assay

5-LOX was purified and assayed by the method described by Reddanna et al.[30] Enzyme activity was measured using polarigraphic method with a Clark's oxygen electrode on Strathkelvin Instruments, model 782, RC-300. Typical reaction mixture contained 50-100 μL of enzyme and 10 μL of substrate (133 μM of AA) in a final volume of 3 mL with 100 mM phosphate buffer pH 6.3. Rate of decrease in oxygen concentration was taken as a measure of enzyme activity. Stock solutions of test compounds, prepared immediately before use, were dissolved in DMSO. Various concentrations of test drug solutions were added and the LOX reaction was initiated by the addition of substrate. The reaction was allowed to proceed at 25 °C and the maximum slope generated was taken for calculating activity. Percent inhibition was calculated by comparison of LOX activity in the presence and absence of inhibitor. The concentration of the test compound causing 50% inhibition (IC_{50}, μM) was calculated from the concentration–inhibition response curve. Each assay was repeated thrice.

4.4. HPLC analysis

HPLC (Shimadzu SPD-6AV) was performed to analyze the inhibitory activity of compound **5e** according to the method described earlier with some modification (Reddanna et al. 1988).[37] In brief, Potato 5-LOX was incubated with AA (133 μM) in 0.1 M potassium phosphate buffer (pH 6.3) for 2 min and the reaction was terminated by acidifying the reaction mixture to pH 3.0 with 6 N HCl. The products formed were extracted with equal volumes of hexane/ether (1:1) twice and the organic solvent was evaporated under inert conditions. The dried products were then dissolved in straight phase HPLC mobile phase consisting of hexane/propane-2-ol/acetic acid in 1000:15:1 ratio and separated on the straight phase analytical column (Type: LUNA 5μ SILICA (2), 250 × 4.60 mm, Phenomenex) at a flow rate of 1 mL/min. Standard 5-HPETE was synthesized as per Reddy et al. (1992).[38] Enzyme without any inhibitor was taken as control. Four micrometer of compound **5e** was taken as test concentration for the study.

4.5. MTT assay

Cell lines used in this study were maintained in tissue culture petri dishes. Medium for the cell line was RPMI-1640 supplemented with 10% heat inactivated fetal bovine serum (FBS) 100 IU/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine. The cell line was maintained in a humidified atmosphere with 5% CO_2 at 37 °C. The cultured cells were passaged twice a week, seeding at a density of 5×10^3 cells per well in 96-well plate before the day of experiment. Before the treatment with test compound, cells were washed with PBS and fresh medium was added. Cell proliferation was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) staining as described by Mosmann et al. (1983).[39] The MTT assay is based on the reduction of the tetrazolium salt, MTT, by viable cells. The NADH or NADPH generated in the living cells, convert the yellow form of the MTT salt to insoluble, purple formazan crystals. In brief, Cells (5×10^3 cells per well) were incubated in 96-well plate in the presence or absence of the test compounds (0.1–100 μM) for 24 h in a final volume of 100 μL. At the end of the treatment, 20 μL of MTT (5 mg/mL in PBS) was added to each well and incubated for an additional 4 h at 37 °C. The purple-blue MTT formazan precipitate was dissolved in 100 μL of DMSO. The activity of the mitochondria, reflecting cellular growth and viability, was evaluated by measuring the optical density at 570 nm on Quant Bio-tek Instruments, Inc. micro titre plate reader. Each concentration was tested in three different experiments run in three replicates.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.06.107. These data include MOL files and InChiKeys of the most important compounds described in this article.

References and notes

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