Received Date : 07-Dec-2012

Revised Date : 10-Apr-2013

Accepted Date : 23-Apr-2013

Article type : Research Article

In-vitro study of isoflavones and isoflavans as potent inhibitors of human 12- and 15-lipoxygenases

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This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as an 'Accepted Article', doi: 10.1111/cbdd.12157

Abstract

In this study, we have investigated sixteen isoflavone and isoflavan derivatives as potential inhibitors of human lipoxygenases (platelet 12-LOX, reticulocyte 15-LOX-1, and epithelial 15-LOX-2). The flavonoid baicalein, a known lipoxygenase inhibitor, was used as positive control. Four compounds, 6,7-dihydroxy-3'-chloroisoflavone (**1c**), 7-hydroxy-8-methyl-4'-chloroisoflavan (**5a**), 7,8-dihydroxy-4'-methylisoflavan (**5b**) and 7,8-dihydroxy-3'-methylisoflavan (**5c**), were effective inhibitors of 12-LOX and 15-LOX-1 with IC_{50}'s less than 10 μM, while 6,7-dihydroxy-4'-nitroisoflavone (**1b**) was a selective inhibitor of 12-LOX. Docking studies, antioxidant assays and kinetic measurements were done for the three best inhibitors (**1b**, **5b**, **5c**). The results showed that a catechol group in ring A is critical for the antioxidant properties of these compounds, and probably essential for their inhibitory activity. Kinetic assays showed that compounds **1b**, **5b**, **5c** are competitive inhibitors with K_i values in the range of 0.3 to 3 μM.

Keywords: *Human lipoxygenase assays, isoflavonoids, docking and scavenger properties*

1. Introduction

Lipoxygenases (LOX's) are a family of non-heme iron-containing dioxygenases which catalyze the stereospecific insertion of molecular oxygen into arachidonic acid [1]; the products formed are the either (*S*)- or (*R*)-enantiomers of hydroperoxy fatty acids, which are further metabolized to the hydroxy derivatives as end products [2]. Mammalian LOX's are classified with respect to the positional specificity with which they oxygenate arachidonic acid and are referred to as 5-, 8-, 12- and 15-LOX [2, 3].

The biological properties of LOXs have been widely studied because they are involved in the biosynthesis of leukotrienes (LT's) and lipoxins (LP's), which participate in different human pathologies. For example,

reticulocyte 15-lipoxygenase (15–LOX-1) has been implicated in colorectal [5] and prostate cancer [6], whereas platelet 12-lipoxygenase (12–LOX) has been implicated in pancreatic [7], breast [8, 9], and prostate cancers [10, 11]. In contrast, epithelial 15-lipoxygenase-2 (15-LOX-2) appears to be anti-tumorigenic in prostate cancer [12]. Inhibition studies of 12-LOX and 15-LOX-1 are directly aimed at the reduction of fatty acid metabolite production associated with inflammatory processes, cell proliferation and carcinogenesis.

The proposed mechanism for fatty acid dioxygenation requires the oxidation of the isolated, inactive, ferrous enzyme to the active ferric form. The ferric ion subsequently abstracts a hydrogen atom from the substrate, with a concomitant reduction of the iron to the ferrous state. Therefore, the oxidation state of LOX is critical to the activity of the enzyme and consequently, the search for compounds that can reduce the ferric ion and therefore lower the activity of LOX, constitutes an important line of research [13, 14 and 15].

Lipoxygenase inhibitors have been divided into five types: reductants, iron chelators, substrate analogs, tyrosine kinase inhibitors and lipoxygenase induction inhibitors [16]. Some examples include hydroxamic acids [17], tropolones [18] and catechols [19]. Numerous flavonoids have been reported over the years as human LOX inhibitors [20, 21, 22], with baicalein (5, 6, 7-trihydroxyflavone) being the most frequently used. Baicalein is a non-specific LOX inhibitor [23], which has been shown to induce apoptosis in breast, prostate, colon, and pancreatic cancer cell lines [23]. Other flavonoids that also inhibit LOXs are fisetin, quercetin and luteolin [24].

In a previous paper we reported that a catechol group in ring A, combined with a fully hydrogenated C ring of an isoflavan backbone, are important features for the activity as lipoxygenase inhibitors [25, 26]. Now we present the evaluation as potential inhibitors of sixteen structurally related isoflavones and isoflavans against platelet 12-LOX, reticulocyte 15-LOX-1 and prostate epithelial 15-LOX-2. Scavenging properties and K_i values were also determined for the most prominent inhibitors (**1b**, **5b** and **5c**). Docking studies were performed to clarify their structure-activity relationship.

2. Materials and methods

The purity of each isoflavonoid compound was confirmed by HPLC using a Merck-Hitachi Intelligent L-6200A Pump, an L-4250 UV-Vis Detector, and a D-7000 HSM System Manager Report, with a C18 reverse phase column (Hypersil ODS-5, 250 mm x 4 mm) at a flow rate of 1 mL/min. The isoflavones were detected at 260 nm, and the isoflavans at 295 nm. Two different solvent systems were used: system 1: (A) acetonitrile and (B) 1% acetic acid, and system 2: (A) acetonitrile and (B) a 1:1 v/v mixture of 1% acetic acid/methanol. In both cases a gradient was used, beginning with 30% of (A), reaching 99% at 30 min, and (B) starting with 70% and ending with 1% in 30 min. All of them showed purity higher than 95%.

2.1. Synthesis of isoflavones and isoflavans

Melting points were recorded on a capillary Microthermal instrument, and were not corrected. All starting materials were commercially available (Sigma-Aldrich), with purity higher than 98%, and used without further purification.

Isoflavones **1**-**5** were prepared by reported procedures [25, 26], from the corresponding intermediate benzylphenylketones.

Preparation of intermediate benzylphenylketones (general procedure) [25, 26] – Dry HCl was passed into a cooled (0 ºC), stirred mixture of the appropriate substituted phenylacetonitrile (0.34 mol) and anhydrous zinc chloride (30 g, 0.22 mol) in dry diethyl ether (200 mL). The corresponding polyhydroxybenzene (0.28 mol) was added portionwise with constant bubbling of gaseous HCl. The reaction mixture was then stirred at room temperature for a few hours. The ketiminium chloride intermediate that separated as an oil was washed with diethyl ether and hydrolyzed by refluxing in 5% HCl (1 L) for 4 to 5 hours. The ketone that separated upon cooling was filtered and recrystallized in the appropriate solvent.

Preparation of isoflavones (general procedure) [25, 26] To a solution of the benzylketone (0.18 mol) in dry DMF (200 mL) was added dropwise $BF_3.Et_2O$ (0.88 mol). To this solution, warmed to 50 ºC, was slowly added a solution of methanesulfonyl chloride (0.56 mol) in DMF (100 mL). The resulting mixture was then heated to 100 ºC for 2 hours. After cooling, it was poured into water (4 L) and left overnight to give a precipitate, which was stirred for 2 hours in cold methanol (50 mL), filtered and crystallized in the appropriate solvent.

In this way the following isoflavones were obtained:

6,7-Dihydroxy-4'-chloro-isoflavone (**1a**). (77%). ^{1}H NMR (DMSO-d_6): δ_H 9.50 (1H, s, 7-OH), 9.06 (1H, s , 6-OH), 8.16 (1H, s, 2-H), 7.48 (2H, d, J = 8.0 Hz, H-3', H-5'), 7.37 (1H, s, H-5), 7.29 (2H, d, J = 8.0 Hz, H-2', H-6'), 6.43 (1H, s, H-8). ^{13}C NMR (DMSO-d_6): δ_C 174.7, 152.4, 151.9, 151.2, 144.7, 133.9, 129.7, 128.6, 123.5, 127.1,116.9, 108.4, 102.8. M.p. 299 °C, lit. [26] M.p. 300 °C;

6,7-Dihydroxy-4'-nitroisoflavone (**1b**). (81%). ^{1}H NMR (DMSO-d_6): δ_H 10.11 (1H, s, 7-OH), 8.54 (1H, s, 2-H), 8.26 (2H, d, J = 8.8 Hz, H-30, H-5'), 7.89 (2H, d, J = 8.8 Hz, H-2', H-6'), 7.40 (1H, s, H-5), 6.93 (1H, s,H-8), [signal for 8-OH not seen]. ^{13}C NMR (DMSO-d_6): δ_C 174.3, 155.5, 153.3, 151.7, 147.2, 145.8, 140.4, 130.5, 123.9, 121.6, 117.1, 108.9, 103.7. M.p. 168 °C, lit. [26] M.p. 166 °C.

6,7-Dihydroxy-3'-chloro-isoflavone **(1c)**. (69 %). ^{1}H NMR (DMSO-d_6): δ_H 10.20 (2H, s, 6-,7-OH), 8.44 (1H, s, H-2), 7.44-7.69 (4H, m, H-2',4',5',6'), 7.40 (1H, s, H-5), 6.96 (1H, s, H-8). ^{13}C NMR (DMSO-d_6): δ_C 174.9, 153.3, 151.9, 151.2, 144.7, 134.1, 130.7, 129.4, 129.1, 128.3, 123.9, 117.1, 108.4, 102.8. M.p. 308 °C, lit. [27] M.p. 306 °C;

7,4'-Dihydroxy-8-methyl-isoflavone **(2a).** (66 %). ^{1}H NMR (DMSO-d_6): δ_H 10.61 (1H, s, 7-OH), 9.86 (1H, s, 4'-OH), 8.13 (1H, s, H-2), 7.97 (1H, d, J = 8.0 Hz, H-5), 7.24 (2H, m, H-2',6'), 7.0 (1H, d, J = 8.0 , H-6), 6.84 (2H, m, H-3',5'), 2.25 (3H, s, 8-CH_3). ^{13}C NMR (DMSO-d_6): δ_C 175.0, 161.5, 157.5, 155.4, 153.5, 130.0, 124.3, 123.8, 117.2, 115.3, 113.9, 110.9, 8.01. M.p. 192 °C , lit. [28] M.p. 193 °C.

7-Hydroxy-8,4'-dimethyl-isoflavone **(2b)**. (71%). ^{1}H NMR (DMSO-d_6): δ_H 10.55 (1H, s, 7-OH), 8.17 (1H, s, H-2), 7.98 (1H, d, J = 8.0 Hz ,H-5), 7.29 (2H, m, H-2',H-6'), 7.19 (2H, m, H-3',5'), 7 (1H, d, J = 8.0 Hz, H-6), 2.45 (3H, s, 4'-CH_3), 2.25 (3H, s, 8-CH_3). ^{13}C NMR (DMSO-d_6): δ_C 173.5, 158.3, 155.4, 153.5, 138.2, 129.1, 128.8, 126.1, 124.2, 123.8, 116.6, 113.9, 110.9, 21.4, 7.9. M.p. 264 °C, lit. [27] M.p. 266 °C.

7-Hydroxy-8-methyl-4'-nitro-isoflavone **(2c)**. (51%). ^{1}H NMR (DMSO-d_6): δ_H 10.85 (1H, s, 7-OH), 8.38 (1H, s, H-2), 8.18 (2H, m, H-3',5'),7.47 (2H, m, H-2',6'), 7.97 (1H, d, J = 8.0 Hz, H-5), 7.00 (1H, d, J = 8.0 Hz, H-6), 2.25 (3H, s, 8-CH_3).^{13}C NMR (DMSO-d_6): δ_C 178.5, 155.2, 153.5, 147.5, 132.00, 130.1, 124.3, 124.1, 123.8, 114.00, 110.9, 8.0. Mp. 345 °C, lit. [27] M.p. 344 °C;

7-Hydroxy-8-methyl-3'-trifluoromethyl-isoflavone **(2d)**. (68%). ^{1}H NMR (DMSO-d_6): δ_H 2.21 (3 H, s, 8-CH_3), 7.10 (1H, d, J = 9.0 Hz, 6-H), 7.64 (1 H, t, J = 8.0 Hz, 5' -H), 7.71 (1 H, d, J = 8.0 Hz, 6' -H), 7.83 (1 H, d, J = 9.0 Hz, 5-H), 7.85 (1 H, d, J = 8.0 Hz, 4' -H), 7.97 (1 H, s, 2' -H), 8.56 (1 H, s, 2-H), 10.68 (1 H, s, 7-OH). ^{13}C NMR (DMSO-d_6): δ_C 174.1, 161.2, 156.6, 153.4, 135.7, 133.1, 132.7, 129.0, 127.2, 126.4, 125.9, 124.8, 124.1, 115.6, 115.5, 112.7, 10.1. Mp. 282 °C, lit. [27] Mp. 283–284 °C.

7,4'-Dihydroxy-isoflavone **(3a)**. (65%). ^{1}H NMR (DMSO-d_6): δ_H 10.90 (1H, s, 7-OH), 9.59 (1H, s, 4`-OH), 8.24 (1H, s, 2-H), 7.96 (1H, d, 5-H), 6.77 (2H, dd, J = 8.4 Hz, 2`-H, 6`-H), 6.34 (1H, s, 8-H), 6.32 (2H, dd, J = 8.4 Hz, 3`-H, 5`-H), 6.16 (1H, d, 6-H). ^{13}C NMR (DMSO-d_6): δ_C 173.3, 162.9, 156.9, 153.0, 130.0, 127.6, 125.0, 122.8, 116.6, 117.0, 115.0, 102. M.p. 300 °C, lit. [29] M.p. 299 °C;

7-Hydroxy-isoflavone **(3b)**. (78%). ^{1}H NMR (DMSO-d_6): δ_H 10.17 (1H, s, 7–OH), 8.18 (1H, s, H-2), 7.97 (1H, d = 7.6 Hz, H-5), 7.55 (5H, m, H-2',3',4',5' and 6'), 6.98 (1H, d, J = 7.6 Hz, H-6), 6.91 (1H, s, H-8). ^{13}C NMR (DMSO-d_6): δ_C 173.25, 162.86, 157.96, 154.03, 133.24, 128.74, 128.69, 128.14, 127.60, 125.00, 117.00, 116.63, 102.14. M.p. 207 °C, lit. [30] M.p. 206 °C.

7-Hydroxy-4'-nitro-isoflavone **(3c)**. (63%). ^{1}H NMR (DMSO-d_6): δ_H 11.01 (1H, s, 7-OH), 8.68 (1H, s, 2-H), 8.35 (2H, dd, J = 7.6 Hz, 3`-H, 5`-H), 8.07 (1H, d, 5-H), 7.97 (2H, dd, J = 7.6 Hz, 2`-H, 6`-H), 7.04 (1H, s, 8-H), 6.99 (1H, d, 6-H). ^{13}C NMR (DMSO-d_6): δ_C 173.3, 162.9, 157.9, 154.0, 148.1, 142.2, 129.3, 127.6, 125.0, 117.0, 116.6, 102.1. M.p. 189 °C, lit. [26] M.p. 188 °C.

7-Benzyl-4'-methoxy-isoflavone **(3d)**. (72%). ^{1}H NMR (DMSO-d_6): δ_H 8.87 (1H, s, H-2), 7.70 (1H, d, J = 7.6 Hz, H-5), 7.33 (2H, d, J = 7.2 Hz, H-2',-6'), 7.24 (5H, m,-AR-H), 6.68 (1H, d,J = 7.6 Hz ,H-6), 6.66 (2H, d, J = 7.2Hz, H-3',5'), 6.65 (1H, s, H-8), 5.17 (2H, s, -OCH_2Bz), 3.77 (3H, s, -OCH_3). ^{13}C NMR (DMSO-d_6): δ_C 173.25, 163.73, 158.89, 156.27, 154.03, 137.09, 129.57, 128.32, 128.16, 126.63, 125.00, 124.05, 120.13, 116.79, 113.94, 103.16, 70.84, 56.04. M.p. 170 °C, lit. [26] M.p. 169 °C;

5,7-Dihydroxy-4'-nitro-isoflavone **(4a)**. (63%). ^{1}H NMR (DMSO-d_6): δ_H 12.66 (1H, s, 5-OH), 10.96 (1H, s, 7-OH), 8.56 (1H, s, 2-H), 8.26 (2H, dd, J = 7,6 Hz, 3`-H, 5`-H), 7.86 (2H, dd, J = 7,6 Hz, 2`-H, 6`-H), 6.40 (1H, s, 8-H), 6.22 (1H, d, 6-H). ^{13}C NMR (DMSO-d_6): δ_C 179.03, 164.88, 161.41, 158.83, 154.71, 148.14, 142.23, 129.33, 125.56, 125.01, 104.92, 99.33, 93.78. M.p. 197 °C, lit. [26] M.p. 196 °C.

5-Hydroxy-7-benzyl-4'-nitro-isoflavone **(4b)**. (72%). ^{1}H NMR (DMSO-d_6): δ_H 11.05 (1H, s, 5-OH), 7.85 (1H, s, H-2), 7.55 (2H, d, J = 7.2 Hz, H-2',6'), 7.23 (5H, m, -Bz), 6.25 (1H, s, H-8), 6.17 (1H , s, H-6), 5.17 (2H , m, O-CH_2-Bz), 6.17 (1H, s, H-6), 6.25 (1H, s, H-8). ^{13}C NMR (DMSO-d_6): δ_C 179.03, 166.46, 161.77, 157.83, 154.71, 148.14, 142.23, 137.09, 129.33, 128.32, 128.17, 128.16, 125.56, 125.01, 106.03, 99.38, 93.94, 70.84.M.p. 185 °C, lit. [27] M.p. 184 °C.

The isoflavans were obtained from the corresponding isoflavones by catalytic hydrogenation with Pd/C (10%) in acetic acid containing 0.1% concentrated sulfuric acid for 14 h [26].

In this way the following isoflavans were prepared:

7-Hydroxy-8-methyl-4'-chloroisoflavan (**5a**). (53%). ^{1}H NMR (DMSO-d_6): δ_H 10.61 (1H, s, 7-OH), 7.37 (2H, d, J=8.4 Hz, H-2',6'), 7.34 (2H, d, J=8.4Hz, H-3',5'), 6.45 (1H, d, J=7.6 Hz, H-5), 6.31(1H, d, J=7.6 Hz, H-6), 2.31 (3H, s, 8-CH_3) ^{13}C NMR (DMSO-d_6): δ_C 157.9, 154.3, 136.6133.0, 129.9, 128.4, 127.4, 117.6, 107.9, 70.3, 38.4, 32.9, 9.2. M.p. 159°C. lit. [27] M.p.160°C.

7,8-Dihydroxy-4'-methylisoflavan (**5b**). (55%). ^{1}H NMR (DMSO-d_6): δ_H 8.54 (1H, s, OH-7), 8.09 (1H, s, OH-8), 7.19 (2H, d, J = 8.1 Hz, H-20, H-6'), 7.12 (2H, d, J = 8.1 Hz, H-30, H-5'), 6.36 (1H, d, J = 8.2 Hz, H-5), 6.28 (1H, d, J = 8.2 Hz, H-6), 4.23 (1H, ddd, J = 3.4 Hz, J = 10.4 Hz, J = 1.7 Hz, H-2e), 3.97 (1H, dd, J = 10.2 Hz; J = 10.2 Hz, H-2a), 3.09 (1H, ddd, J = 4.0 Hz, J = 5.7 Hz, J = 10.0 Hz, J = 8.9 Hz, H-3), 2.89 (1H, dd, J = 10.4 Hz, J = 16.1 Hz, H-4a), 2.80 (1H, ddd, J = 5.4 Hz, J = 15.6 Hz, J = 1.2 Hz, H-4e). ^{13}C NMR (DMSO-d_6): δ_C 144.7, 143.6, 139.5, 136.5, 133.7, 129.7, 127.9, 119.1, 113.9, 108.7, 70.5, 38.2, 32.0, 21.3. M.p.136°C, lit. [27] M.p.137°C;

7,8-Dihydroxy-3'-methylisoflavan (**5c**). (55%). ^{1}H NMR (DMSO-d_6): δ_H δ 9.20 (1H, s, 8-OH), 8.60 (1H, s, 7-OH), 7.28 (1H, t, J = 7.4 Hz, H-5'), 7.17 (1H, d, J = 7.4 Hz, H-6'), 6.96 (1H, d, J = 7.6 Hz, H-4'), 6.38 (1H, d, J = 7.4 Hz, H-6), 6.19 (1H, d, J = 7.4 Hz, H-5), 4.54 (1H, dd, J = 12.4, 6.7 Hz, H-2a), 4.23 (1H, dd, J = 12.3, 6.6 Hz, H-2b), 3.60–3.47 (1H, m, H-3), 3.14 (1H, ddd, J = 12.5, 8.5, 1.0 Hz,H-4a), 2.90 (1H, ddd, J = 12.3, 8.5, 1.0 Hz, H-4b), 2.27 (3H, s, 3'-CH_3). ^{13}C NMR (DMSO-d_6): δ_C 146.9, 146.0,

141.7, 137.6, 133.0, 129.2, 128.4, 126.4, 126.1, 123.1, 113.9, 70.3, 34.7, 32.9,21.4. M.p.268°C, lit. [27] M.p.265°C.

2.2. **Kinetics and IC_{50} assay**

The H_6-tagged proteins, 12-LOX, 15-LOX-1 and 15-LOX-2, were expressed and purified as described previously [31, 32, and 33]. The inhibition of their activities was determined by following the formation of the conjugated diene product at 234 nm (ε = 25,000 $M^{-1}cm^{-1}$) with a Perkin-Elmer Lambda 40 UV/Vis spectrophotometer, relative to control rates of carrier solvent DMSO as previously published [34]. All reactions were done in a volume of 2 mL constantly stirred using a magnetic stir bar at room temperature (23 ºC) with approximately 2.040 Units of 12-hLOX (evaluated by iron content), 4.200 Units 15-hLOX-1 (by iron content), and 6.600 Units of 15-hLOX-2. Units of specific activity were defined as (µmol substrate consumed/ mg enzyme x min). Reactions with 12-hLOX were carried out in 25 mM HEPES (pH 8.0), 0.01% Triton X-100 and 10 µM arachidonic acid (AA). Reactions with 15-hLOX-1 and 15-hLO-2 were carried out in 25 mM HEPES buffer (pH 7.5), 0.01% Triton X-100 and 10 µM AA and 30 µM AA, respectively. The concentration of AA (for 12-hLOX and 15-hLOX-2) was quantitatively determined by allowing the enzymatic reaction to reach completion. IC_{50} values were obtained by determining the enzymatic rate at various inhibitor concentrations and plotted against inhibitor concentration, followed by a hyperbolic saturation curve fit. The data used for the saturation curves were obtained in duplicate or triplicate, depending on their quality. Additionally the K_i and inhibitor behavior of the three most interesting compounds (**1b**, **5b,** and **5c**) were determined against soybean 15-LOX (specific activity 100000 U/mg, Cayman Chemical Catalog Number 60712) using 10 µM AA as substrate in Hepes-Triton X-100 buffer, pH 7.4. The reaction velocities at different inhibitor concentration expressed as logarithm, (0-50µM) in triplicate for three independent experiments for each compound were plotted

and the K_i values were interpolated from the Cheng-Prusoff [35] equation, using GraphPad Prism v.5 DEMO [36]

2.3. **Evaluation of radical-trapping activity using DPPH**

The DPPH scavenging capacity assay was performed using 20 µL of different compound concentrations in methanol, which were added to 1 mL of 0.1 mM DPPH in methanol. Each mixture was vortexed for a few seconds and left with agitation in the dark for 30 min at room temperature. The absorbance (*A*) of each reaction mixture was measured at 517 nm against a blank of methanol using a UV-visible spectrometer (Genesys 5). The scavenging capacity of each sample was calculated on the basis of an ascorbic acid standard curve. Duplicate reactions were carried out with each sample [37]. The correlation among the IC_{50} values for enzyme inhibition and the scavenger properties of compounds was evaluated using a correlation matrix including the IC_{50} for enzyme inhibition of 12 compounds and the percentage of remaining DPPH radical for 3 independent experiments with each enzyme. The Pearson correlation for the IC_{50} and % remaining DPPH was done using GraphPad Prism v.5 DEMO, considering an alpha value of 0.05.

2.4. **Docking studies**

All structures were built with the GaussianView software [38]. The Chelpg charges were obtained at the B3LYP/6-31G** level of theory, employing the Gaussian 03 package [38]. Docking into the active site of human 12- and 15-lipoxygenase models and the crystal structure of soybean LOX-1 (PDB ID: 1N8Q) [39] was done using AutoDock 4.2 [40] with the Lamarckian algorithm and assuming total flexibility of the inhibitors and partial flexibility of the His residues coordinated to Fe^{+3} inside the binding site. The grid maps were built with 60 x 60 x 60 points, with a grid-point spacing of 0.375 Å. The AutoTors option

was used to define the ligand torsions, and the docking results were then analysed by a ranked cluster analysis, resulting in conformations with the highest overall binding energy (most negative $-\Delta G_{binding}$ value).

3. **Results and Discussion**

The studied isoflavones were prepared by a two-step procedure. Reduction of the isoflavones by catalytic hydrogenation led to the corresponding isoflavans, as shown in Scheme 1.

Table 1 shows the sixteen isoflavonoids that were investigated as potential inhibitors of human platelet 12-LOX, reticulocyte 15-LOX-1 and epithelial 15-LOX-2. The compounds evaluated were thirteen isoflavones, three isoflavans and the flavone baicalein as a positive control [23].

Compounds **1a** and **1b,** with a catechol group on ring A and a chloro or nitro group at the *para* position of B ring, respectively, had IC_{50} values >100 μM toward 15-LOX-1. On the other hand, **1b** proved to be a selective inhibitor of platelet 12-LOX (IC_{50} 13 μM). In addition to the inhibitory assays, docking studies were performed for all compounds based on human 12-LOX and 15-LOX-1 models. In 12-LOX, the nitro group of **1b** is positioned close to Arg402 and Gln406 (1.79 Å and 1.78 Å, respectively), and also near the exit of the binding site, enabling electrostatic interactions. Ring B is parallel to Ile399 in the enzyme cavity, allowing hydrophobic stabilization of this aromatic moiety, as has been reported for soybean LOX [41] (Figure 1). In 15-LOX-1, the small, neutral aminoacid Gly replaces the polar, ionic Gln406, which might be an important modification to explain the weak inhibitory effect of **1b** on this enzyme.

Compound **1c** with a chloro substituent in the *meta* position of ring B inhibits 12-LOX and 15-LOX-1 with IC_{50} values of 0.28 and 0.59 μM, respectively. The analysis of its interaction in the binding cavity of 12-LOX indicates that the *meta* chloro group in **1c** participates in a stabilizing hydrophobic interaction with Leu172, unlike the behavior of ring B in compound **1a** with a *para* chloro group. In addition, ring B of **1c** can rotate in the active site of 15-LOX-1 producing more favorable hydrophobic interactions, similar to those recently reported for other inhibitors [42]. This difference in inhibitory activity towards LOX might be due to the different shape and size of the binding cavity [44] of the enzyme isoforms. This effect was observed with compounds **1b** and **1c** against 12-LOX, 15-LOX-1 and 15-LOX-2 (see Table 1).

Compounds **2a**, **2b**, **2c** and **2d,** were unable to inhibit 12-LOX and 15-LOX-1 (IC_{50} > 100 μM). This could be due to the presence of the methyl group on C-8 of ring A and the availability of a single hydroxyl group instead of a catechol group to coordinate with the iron atom in the active site. Other studies have reported that catechol moieties as found in many lipoxygenase inhibitors are an important structural features [41]. The evaluation of **3a**, **3b**, **3c** and **3d,** which likewise do not contain a catechol ring, also revealed no inhibitory activity against 12-LOX and 15-LOX-1, confirming the importance of the catechol group on ring A. Besides, the evaluation of compounds **4a** and **4b** exhibiting low activities against human LOX (IC_{50} >100 μM) also showed that one OH group attached to C-7 on ring A is not sufficient to make a compound a good inhibitor.

Following with our study, the biological evaluation of isoflavan derivatives showed that these compounds were effective but not selective for the three lipoxygenases studied. The IC_{50} values were 4.4 (**5b**), 5.8 (**5c**) and 29 (**5a**) μM for 15-hLOX-1 and 6.4 (**5c**) 7.7 (**5b**) and > 40 (**5a**) μM for human 12-LOX. The analysis of the inhibitory activity of these isoflavans revealed decreased potency of compound **5a** versus compounds **5b** and **5c**. This behavior could be explained by the presence of a methyl group at C-8 instead of a hydroxyl group in **5a**, so that the presence of a catechol moiety on ring A is prevented. Furthermore,

isoflavans that have a fully hydrogenated ring C are able to fit more efficiently into the binding site of human LOXs and consequently proved to be better inhibitors. Also, we found that the IC_{50} value observed for **5b** against 15-LOX-2 (34 μM) is eight times higher than its IC_{50} value against 15-LOX-1 (4.4 μM), 15-LOX-1 favoring cancer development while 15-LOX-2 had shown anti-tumor activity [12].

The antioxidant capacity of twelve compounds was estimated by reaction with DPPH [37] and the results are summarized in table2.

The compounds without catechol moieties, which did not show LOX inhibition, were also unable to bleach the DPPH radical. Although only 12 of 16 compounds were evaluated, a good correlation was observed among the IC_{50}s for enzyme inhibition and the IC_{50}s for the reduction of DPPH ($P = 0.0002$ and $P = 0.0042$ for 12-LOX and 15LOX-1, respectively). These results support the previous evidence that there is a close relationship between the inhibitory capacity of lipoxygenases by catechol isoflavonoids and the antioxidant properties of the latter [26]. Because of the great similarity among the active sites of the different lipoxygenases, kinetic studies were performed with three inhibitors (**1b**, **5b** and **5c**) to determine if the isoflavonoids were competitive inhibitors of soybean lipoxygenase, as a model of the different LOXs. All compounds assayed proved to be competitive inhibitors with low *K*i, especially isoflavans **5b** and **5c**, with *K* an order of magnitude lower than **1b** (3.4 , 0.53 and 0.25 μM), as shown in Figure 2 and summarized in table 3 [43].

The docking studies also show the most favorable interactions between the binding site of soybean LOX and compounds **1b**, **5b** and **5c**, as shown in Figure 3.

Conclusion

Sixteen isoflavonoids were evaluated as potential inhibitors of lipoxygenases. We found that catechol groups and the flexibility of ring C were important features for higher inhibitory capacity of these compounds, as can be observed particularly in isoflavones **1b**, **1c** and isoflavans **5b** and **5c**, which proved to be potent and selective inhibitors of the human isoforms 12- and 15-LOX-1. Additionally, the presence of a chlorine atom at the *meta* instead of the *para* position on ring B enhances the inhibitory effect of the isoflavonoids toward LOX. Because various studies have suggested a protective role of 15-hLOX-2 against cancers, we have included this enzyme in our study, butit was not inhibited by any of the tested compounds. Also in this work, kinetic assays were performed for **1b**, **5b** and **5c**, showing that all three are competitive inhibitors. Another important feature observed was the direct correlation between the inhibition of 12- and 15-hLOX1 and the antioxidant capacity of the inhibitors, determined by reaction with DPPH. Finally docking studies supported the experimental evidence. Therefore, this paper confirm the previously results [26] concerning the importance of the catechol group and reduction of the central ring on the inhibition of 12 - and 15-lipoxygenase and alsowas observed that a chlorine atom in position 3' of the B ring of the isoflavonoid improved the capacity as lipoxygenase inhibitor as seen in 1c.

Acknowledgments

Financial support from FONDECYT (project # 1120379) is gratefully acknowledged.

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Figure and Scheme Captions

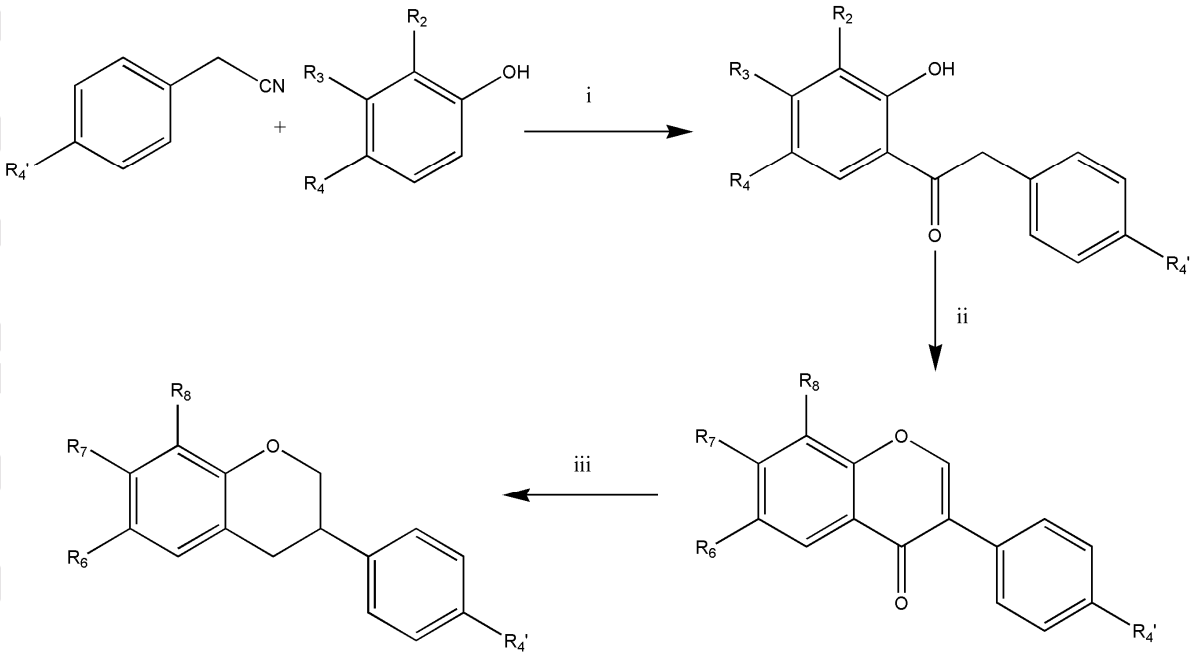
Scheme 1. General route for the preparation of isoflavones and isoflavans.

Figure 1. Principal interactions of isoflavonoid **1b** and the binding site of human 12-LOX

Figure 2. Relation between Log inhibitor concentrations and reaction velocity (u.a/min) of soybean 15-LOX and three inhibitors

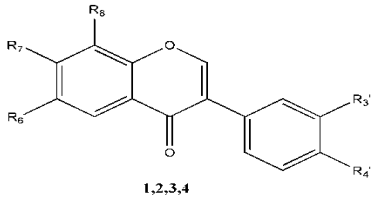
Figure 3. Superimposition and most important interactions of **1b** (green), **5b** (light blue) and **5c** (magenta) in the binding site of soybean LOX by docking studies

Scheme 1

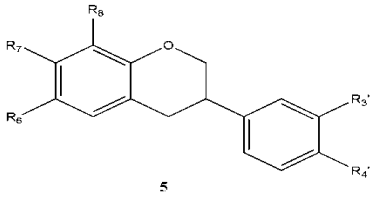


(i)$ZnCl_2/H_2O/H^+$; (ii) DMF/$MeSO_2Cl$ in BF_3OEt_2; (iii) H_2/Pd/C/HAc-0.1%conc. H_2SO_4

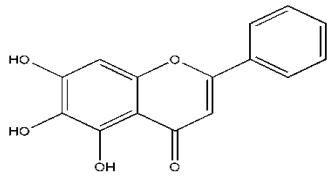
Table 1. Structures of isoflavonoids and baicalein as inhibitors of human lipoxygenases and their inhibitory activity (IC_{50}) against platelet 12-LOX, reticulocyte 15- LOX-1, epithelial 15-LOX-2.



1,2,3,4



5



Baicalein

Compounds	12-LOX [μM]	15-LOX-1 [μM]	15-LOX-2 [μM]	R_5	R_6	R_7	R_8	R_3'	R_4'
1a	**>100**	**>100**	**N.D**	**H**	**OH**	**OH**	**H**	**H**	**Cl**
1b	**13**	**>100**	**>30**	**H**	**OH**	**OH**	**H**	**H**	**NO_2**
1c	**0.28**	**0.59**	**>30**	**H**	**OH**	**OH**	**H**	**Cl**	**H**
2a	**>100**	**>100**	**N.D**	**H**	**H**	**OH**	**CH_3**	**H**	**OH**
2b	**>100**	**>100**	**N.D**	**H**	**H**	**OH**	**CH_3**	**H**	**CH_3**
2c	**>100**	**>100**	**N.D**	**H**	**H**	**OH**	**CH_3**	**H**	**NO_2**
2d	**>100**	**>100**	**N.D**	**H**	**H**	**OH**	**CH_3**	**H**	**CF_3**
3a	**>100**	**>100**	**N.D**	**H**	**H**	**OH**	**H**	**H**	**OH**
3b	**>100**	**>100**	**N.D**	**H**	**H**	**OH**	**H**	**H**	**H**
3c	**>100**	**>100**	**N.D**	**H**	**H**	**OH**	**H**	**H**	**NO_2**
3d	**>100**	**>100**	**N.D**	**H**	**H**	**OBn**	**H**	**H**	**OCH_3**

4a	**>100**	**>40**	**N.D**	**OH**	**H**	**OH**	**H**	**H**	**NO_2**
4b	**>100**	**>100**	**N.D**	**OH**	**H**	**OBn**	**H**	**H**	**NO_2**
5a	**>40**	**29**	**N.D**	**H**	**H**	**OH**	**CH_3**	**H**	**Cl**
5b	**7.7**	**4.4**	**34**	**H**	**H**	**OH**	**OH**	**H**	**CH_3**
5c	**6.4**	**5.8**	**33**	**H**	**H**	**OH**	**OH**	**CH_3**	**H**
Baicalein	**3.3**	**11.5**	**71**						

***N.D: Not Determined**

Table 2. Radical DPPH scavenger properties of twelve compounds. Activity expressed as percentage of remaining radical.

Compound	% DPPH remaining
1b	**17.2**
1c	**4.12**
2a	**Without activity**
2b	**Without activity**
2c	**Without activity**
2d	**Without activity**
3a	**Without activity**
3b	**Without activity**
3c	**Without activity**
4a	**Without activity**
5b	**8.41**
5c	**6.7**

Table 3. K_i for inhibition of soybean 15-LOX by compounds **1b**, **5b**, and **5c** obtained by nonlinear regression for one site binding fit of Cheng-Prusoff equation, considering K_m = 13 µM [43] and 10 µM substrate.

Compound	K_i (µM)	R^2	Inhibition type
1b	3.434	0.8683	Competitive
5b	0.5290	0.9737	Competitive
5c	0.2455	0.9536	Competitive