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Research Article

Enzymatic Studies of Isoflavonoids as Selective and Potent Inhibitors of Human Leukocyte 5-Lipo-Oxygenase

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Continuing our search to find more potent and selective 5-LOX inhibitors, we present now the enzymatic evaluation of seventeen isoflavones (IR) and nine isoflavans (HIR), and their in vitro and in cellulo potency against human leukocyte 5-LOX. Of the 26 compounds tested, 10 isoflavones and 9 isoflavans possessed micromolar potency, but only three were selective against 5-LOX (IR-2, HIR-303, and HIR-309), with IC_{50} values at least 10 times lower than those of 12-LOX, 15-LOX-1, and 15-LOX-2. Of these three, IR-2 (6,7-dihydroxy-4-methoxy-isoflavone, known as texasin) was the most selective 5-LOX inhibitor, with over 80-fold potency difference compared with other isozymes; Steered Molecular Dynamics (SMD) studies supported these findings. The presence of the catechol group on ring A (6,7-dihydroxy versus 7,8-dihydroxy) correlated with their biological activity, but the reduction of ring C, converting the isoflavones to isoflavans, and the substituent positions on ring B did not affect their potency against 5-LOX. Two of the most potent/selective inhibitors (HIR-303 and HIR-309) were reductive inhibitors and were potent against 5-LOX in human whole blood, indicating that isoflavans can be potent and selective inhibitors against human leukocyte 5-LOX in vitro and in cellulo.

Key words: human lipo-oxygenase, IC_{50} values, structure–activity relationship, isoflavans derivative, steered molecular dynamics

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In response to extracellular signals, arachidonic acid (AA) is released from the cell membrane producing an intracellular cascade of events, including the activation of lipo-oxygenases (LOX) and cycloxygenases (COX) (1,2), whose products generate pain, fever, and inflammation (3,4). Two such products of LOX, leukotriene (LT) and lipoxin (LP), participate in different physiologic and pathologic process (5–7). 5-LOX catalyzes the synthesis of LTs, by sequential reactions on AA to produce 5-hydroperoxyeicosatetraenoic acid (5-HpETE) and then leukotriene A_4 (LTA_4), the latter being converted to leukotriene B4 (LTB_4) by LTA_4 hydroxylase (8). These lipid mediators are involved in inflammatory and allergic diseases, such as asthma, rhinitis, ulcerative colitis, cardiovascular disease, diabetes, metabolic syndrome and also in some types of cancers (9,10). For these reasons, inhibition of 5-LOX is an important target for the treatment of these pathologies.

Previously, we had investigated the inhibitory activity of isoflavonoids on 5-LOX using a suspension of porcine blood leukocytes (11), and the relationship between structure and potency showed that isoflavans were more effective inhibitors than their corresponding isoflavones. The data were rationalized on the basis of the conformational change of ring C after hydrogenation and the interruption of the fully conjugated system. Subsequently, we investigated the effect of isoflavonoids on purified human lipo-oxygenases (platelet 12-LOX, reticulocyte 15-LOX-1, and epithelial 15-LOX-2) (12) and determined that aromaticity and the oxidation state of the isoflavonoid ring C were important for both inhibitory potency and selectivity. The isoflavones and isoflavanones preferentially inhibited 12-LOX, whereas the isoflavans preferentially inhibited 15-LOX-1. Simple modifications of the basic isoflavonoid structure produced a number of selective inhibitors of both LOX, indicating that the isoflavonoid skeleton is a viable scaffold for selective inhibitor development. Recently, we reported results supporting our earlier work and indicating that the catechol moiety and the flexibility of ring C are important features for increased inhibitor potency, with this new group of isoflavans being more potent and selective against human 12-LOX and 15-LOX-1 (13).

In an effort to discover potent and selective 5-LOX inhibitors, we now present the *in vitro* and *in cellulo* potency

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evaluation of 26 related isoflavones (**IR**) and isoflavans (**HIR)** against human leukocyte 5-LOX. In addition, pseudo-operoxidase assays, docking, and steered molecular dynamics (SMD) studies were carried out for the most selective inhibitors to elucidate the relationship between the structural features of the isoflavonoids and their 5-LOX inhibitory potency.

Methods and Materials

Synthesis of isoflavones and isoflavans

All starting materials were commercially available (Sigma-Aldrich, St. Louis, MI, USA), with purity higher than 98%, and were used without further purification. The isoflavonoids were obtained by classic electrophilic substitution of appropriate phenols with benzyl cyanides (*Houben-Hoesch reaction*). The resulting hydroxybenzylketones were cyclized to the isoflavones using DMF/$MeSO_2Cl$ as a carbon atom donor in the presence of BF_3Et_2O. The isoflavans were obtained by catalytic hydrogenation of the corresponding isoflavones with Pd/C (10%) in acetic acid containing 0.1% concentrated sulfuric acid as shown in Schemes 1.

To verify the purity of each compound, HPLC analyses were performed using a Merck-Hitachi Intelligent L-6200A Pump, an L-4250 UV-Vis Detector, and a D-7000 HSM System Manager Report, a C18 reverse phase column (Hypersil ODS-5, 250 × 4 mm), and a flow rate of 1 mL/min. The isoflavones (IR) were detected at 260 nm, and isoflavans (HIR) at 295 nm. Two different solvent systems were used as follows: system 1: (**A**) acetonitrile and (**B**) 1% acetic acid and system 2: (**A**) acetonitrile and (**B**) a 1:1 mixture of 1% acetic acid/methanol. A gradient of 30 min of duration was used in both cases, beginning with 30% of (**A**), reaching 99% at 30 min, and (**B**) starting with 70% and ending with 1% in 30 min. Melting points were recorded using a capillary Microthermal instrument and were not corrected. The purity of all compounds evaluated was higher than 95%. The isoflavones (**IR**) were prepared by reported procedures (12,14,15) from the corresponding intermediate benzylphenylketones.

Preparation of intermediate benzylphenylketones (general procedure)

Dry HCl was passed into a cooled (0 °C), stirred mixture of the substituted phenylacetonitrile (0.34 mol) and anhydrous zinc chloride (30 g, 0.22 mol) in dry diethyl ether (200 mL) (12,14). 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 was separated as an oil, washed with diethyl ether, and hydrolyzed by refluxing in 5% HCl (1 L) for 4–5 h. The ketone that separated upon cooling was filtered and recrystallized in the appropriate solvent.

Preparation of isoflavones (general procedure)

To a solution of the benzylphenylketone (0.18 mol) in dry DMF (200 mL), $BF_3.Et_2O$ was added dropwise (0.88 mol) (12,14). This solution was then warmed to 50 °C and a solution of methanesulfonyl chloride (0.56 mol) in DMF (100 mL) was slowly added. The resulting mixture was then heated to 100 °C for 2 h. After cooling, it was poured into water (4 L) and left overnight to give a precipitate, which was stirred for 2 h in cold methanol (50 mL), filtered, and crystallized in the appropriate solvent. The isoflavones were characterized by standard characterization in supporting information (Data S1).

Expression and purification of human 5-lipoxygenase

5-LOX was expressed as a non-tagged protein and used as a crude ammonium sulfate protein fraction (17,18), while 15-LOX-2 was expressed and purified as a his-tagged protein, as published previously (19).

IC_{50} assay

The inhibition percentages were determined by following the formation of the conjugated diene product at 234 nm (ε = 25 000 M/cm) with a Perkin Elmer (Santa Clara, CA, USA) Lambda 40 UV/Vis spectrophotometer relative to control rates of carrier solvent DMSO as previously published. It is important to mention that all tested isoflavonoids showed absorbance between 255 and 320 nm (18,19). The reactions were done in a volume of 2 mL and constantly stirred using a magnetic stir bar at room temperature (23 °C). Reactions with the crude, ammonium sulfate precipitated 5-LOX were carried out in 25 mM HEPES (pH 7.3), 0.3 mM $CaCl_2$, 0.1 mM EDTA, 0.2 mM ATP, 0.01% Triton-X-100, and 10 μM AA. The concentrations of AA for 5-LOX assays were quantitatively determined by allowing the enzymatic reaction to go to 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.

Pseudoperoxidase activity assay

The reductive properties of the compounds were determined by monitoring the pseudo-peroxidase activity of 5-LOX in the presence of the inhibitor and 13-HPODE (20). Activity was characterized by direct measurement of the product degradation following the decrease of absorbance at 234 nm using a Perkin Elmer Lambda 45 UV/Vis spectrometer (50 mM sodium phosphate (pH 7.4), 0.3 mM $CaCl_2$, 0.1 mM EDTA, 0.01% Triton-X-100, and 10 μM 13-HPODE). All reactions were performed in 2 mL of buffer and constantly stirred with a rotating stir bar (22 °C). Reaction was initiated by addition of 10 μM inhibitor (1:1 ratio to product), and a positive result for activity reflected

a loss of more than 40% of product absorption using zileuton, a known reductive inhibitor. Individual controls were conducted with inhibitor alone with product and enzyme alone with product. These negative controls formed the baseline for the assay, reflecting non-pseudoperoxidase dependent hydroperoxide product decomposition. To rule out the auto-inactivation of the enzyme from pseudo-peroxidase cycling, 15-LOX-1 residual activities were measured after the assay was complete, 20 μM AA was added to the reaction mixture and the residual activity was determined by comparing the initial rates with inhibitor and 13-(S)-HPODE versus inhibitor alone, as the inhibitor by itself inherently lowers the rate of the oxygenation. Activity is characterized by direct measurement of the product formation with the increase of absorbance at 234 nm.

COX-1 and COX-2 inhibition

Ovine COX-1 (cat. no. 60100) and human COX-2 (cat. no. 60122) were purchased from Cayman Chemical. Approximately 2 μg of either COX-1 or COX-2 was added to buffer containing 100 μM AA, 0.1 M Tris–HCl buffer (pH 8.0), 5 mM EDTA, 2 mM phenol, and 1 μM hematin at 37 °C. Data were collected using a Hansatech DW1 oxygen electrode chamber. Inhibitors were incubated with the respective COX isozyme for 20 min and added to the reaction mixture, and the rate of oxygen consumption was recorded. Ibuprofen, aspirin, and the carrier solvent, DMSO, were used as positive and negative controls, respectively (21).

LTB_4 inhibition assay

Whole human blood was purchased through Innovative Research. Blood was dispensed in 150 μL samples followed by addition of inhibitor or control (the DMSO vehicle), and then incubation for 15 min at 37 °C. Blood coagulation was then stimulated by introduction of calcium ionophore A23817 (freshly diluted from a 50 mM stock solution in DMSO to 1.5 mM in Hanks' balanced salt solution (HBSS)) along with incubation for 30 min at 37 °C. Samples were then centrifuged at 7500 × *g* (300 × *g*) for 10 min at 4 °C. Plasma was then separated and diluted (1:100) with HBSS for LTB_4 detection using an ELISA detection kit (Cayman). Inhibitors were added at 10 or 15 μM concentrations (0.5 μM for control compound setileuton), and IC_{50} values were generated using a one point IC_{50} estimation equation. Drug efficacy was determined using a minimum of two different donors (22).

Docking and steered molecular dynamics studies

The 5-LOX structure was built with the GaussianView software (23). ChelpG charges were obtained at the B3LYP/6-31G** level of theory, employing the Gaussian 03 package (23). Docking of all inhibitors into the active site of the crystal structures of human 5-lipo-oxygenase (PDB code: 3O8Y, 2.39 Å resolution) and porcine leukocyte 12-lipo-oxygenase (PDB code: 3RDE, 1.89 Å resolution) (24,25) was performed with the AutoDock4 package (26), using a Lamarckian algorithm and assuming total flexibility of the inhibitors and partial flexibility of the His residues co-ordinated to Fe^{3+} inside the binding site. The grid maps were made up of 60 × 60 × 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 analyzed by a ranked cluster analysis, resulting in conformations with the highest overall binding energy (most negative $-\Delta G_{binding}$ value).

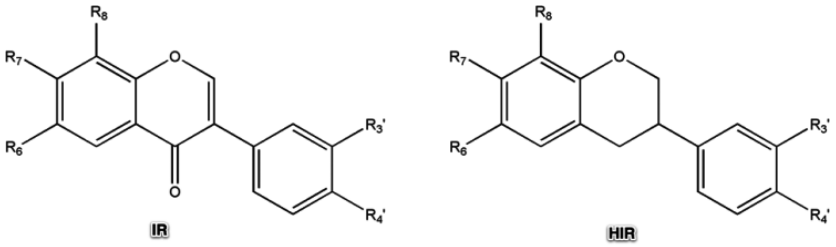
The SMD simulations were performed using NAMD 2.6 (27) with the Charmm33b1 (28) force field. Each 12- and 5-lipo-oxygenase systems with IR-2 were put into a water box 100 × 100 × 100 and 90 × 120 × 110 $Å^3$, respectively, with a layer of water of at least 15 Å in each dimension. Both systems were neutralized, and a cutoff of 10 Å for non-bonded interactions was applied. We performed 250 ps of water equilibration, 10 000 steps of minimization and 50 ps of heating from the 0 K up to 300 K before each main SMD simulation. For IR-2, the 1,2 -ns-long SMD simulations were carried out. The compound was pulled out of the binding site and entered the solvent region. The external force was attached to the center of mass of the molecule, and the velocity of the SMD reference point was 0.00005 Å/ps in both enzymes, and the spring constant was 4 kcal/(mol-A^2), the iron insight into the binding site was fixed to calculate the direction of the vector. To obtain a better statistical result, we repeated all simulations five times. The temperature was kept constant using the Langevin method (310 K). All graphical analysis was performed using the VMD software (29).

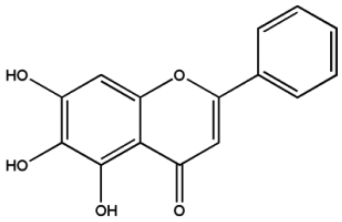
Results and Discussion

Twenty-six isoflavone (**IR**) and isoflavan (**HIR**) derivatives were analyzed as potential inhibitors of human leukocyte 5-LOX (Table 1), of which nineteen proved to have IC_{50} values lower than 10 μM. For comparison, baicalein (the flavone 5,6,7 trihydroxy-2-phenyl chromen-4-one) showed an IC_{50} of 0.85 μM (30). The interpretation of the structure–function relationship was focused on three structural aspects, the catechol group on ring A (6,7-dihydroxy or 7,8-dihydroxy), the oxidation state of ring C (i.e. isoflavones versus isoflavans), and finally the position and chemical features of substituents on ring B.

The role of the catechol moiety in the LOX inhibitors was previously investigated (11–13,31), but the impact of its position for IR (isoflavones) and HIR (isoflavans) derivatives on the biological activity against human 5-LOX has not been fully discussed. Of the current 26 derivatives, only the compounds that possessed a catechol moiety manifested submicromolar potency, indicative of either a

Table 1: Structures of isoflavonoids and baicalein and their inhibitory activity (IC_{50}) against human leukocyte 5-LOX and human 15-LOX type 2





Baicalein

Name	R6	R7	R8	R3′	R4′	12-LOX (12,13) [μM ± SD]	15-LOX-1 (12,13) [μM ± SD]	15-LOX-2 [μM ± SD]	5-LOX [μM ± SD]
IR-1	OH	OH	H	H	OH	8.7 ± 1	49 ± 8	>100	>40
IR-2	OH	OH	H	H	OCH_3	>100	>100	>100	1.2 ± 0.2
IR-3	OH	OH	H	H	CH_3	2.3 ± 0.3	18 ± 7	–	0.36 ± 0.3
IR-4	OH	OH	H	H	H	>40	>40	–	>40
IR-5	OH	OH	H	H	Cl	>100	>100	–	31
IR-6	OH	OH	H	H	NO_2	5.8 ± 0.5	>100	>30	1.5 ± 0.5
IR-8	OH	OH	H	Cl	H	0.28 ± 0.3	0.59 ± 0.07	>30	0.39 ± 0.05
IR-10	OH	OH	H	F	H	4.2 ± 0.5	12 ± 3	–	0.49 ± 0.2
IR-201	H	OH	CH_3	H	OH	>100	>100	–	12 ± 2
IR-203	H	OH	CH_3	H	CH_3	>100	>100	–	1.5 ± 0.3
IR-206	H	OH	CH_3	H	NO_2	>100	>100	–	300
IR-213	H	OH	CH_3	CF_3	H	>100	>100	–	600
IR-301	H	OH	OH	H	OH	–>100	50 ± 20	–	0.9 ± 0.1
IR-303	H	OH	OH	H	CH_3	1.6 ± 0.3	7.8 ± 0.8	>100	0.25 ± 0.05
IR-308	H	OH	OH	Cl	H	0.78 ± 0.08	6.2 ± 0.7	>100	0.3 ± 0.03
IR-309	H	OH	OH	CH_3	H	3.6 ± 0.3	11 ± 0.7	>100	0.3 ± 0.03
IR-406	H	OH	H	H	NO_2	>100	>100	–	5 ± 0.5
HIR-1	OH	OH	H	H	OH	17 ± 1.7	0.5 ± 0.1	71 ± 30	0.3 ± 0.05
HIR-3	OH	OH	H	H	CH_3	5.5 ± 0.6	2.3 ± 0.7	–	0.28 ± 0.09
HIR-7	OH	OH	H	O-CH_2-O	11 ± 1.2	0.35 ± 0.06	16 ± 2	0.4 ± 0.08	
HIR-9	OH	OH	H	CH_3	H	15 ± 1.4	0.21 ± 0.02	8.3 ± 0.9	0.5 ± 0.1
HIR-11	H	OH	OH	OCH_3	OCH_3	17 ± 4	11 ± 3	–	0.69 ± 0.2
HIR-205	H	OH	CH_3	H	Cl	>40	29 ± 5	–	7.1 ± 0.9
HIR-301	H	OH	OH	H	OH	>40	>40	–	0.31 ± 0.06
HIR-303	H	OH	OH	H	CH_3	7.7 ± 1.3	34 ± 0.5	34 ± 7	0.18 ± 0.2
HIR-309	H	OH	OH	CH_3	H	6.4 ± 1	5.8 ± 0.9	33 ± 7	0.15 ± 0.01
Baicalein						0.86 ± 0.3	9.1 ± 0.8	>100	0.85 ± 0.2

chelative or reductive inhibitory mechanism. Of the IRs, the 7,8-catechols (IR-301 and IR-303) were more active than the related 6,7-catechols (IR-1 and IR-3, respectively). On the other hand, the HIRs exhibited little difference when the catechol position was changed (6,7-dihydroxy versus 7,8-dihydroxy), as seen with the comparable potency of HIR-1, -3 and -9 and HIR-301, -303 and 309. These results suggested that changes in the catechol position had larger effects on potency for the IR derivatives than the HIR derivatives, due to the tight binding constraints for isoflavone (*vide infra*). Nevertheless, this difference is minor considering that the most potent IR and HIR derivatives have comparable IC_{50} values, regardless of the position of the catechol.

Among the non-catechol derivatives, only IR-203 (IC_{50} 1.5 μM) and IR-406 (IC_{50} 5 μM) inhibited 5-LOX, with neither having submicromolar potency, indicating the need of

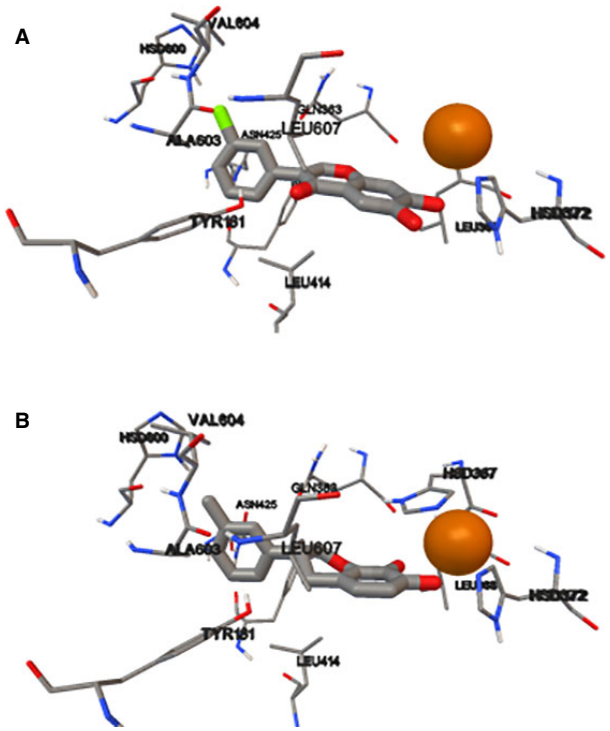


Figure 1: Docking results of IR-8 (A) and HIR-309 (B) into binding site of crystal structure of human 5-LOX (PDB ID 3O8Y).

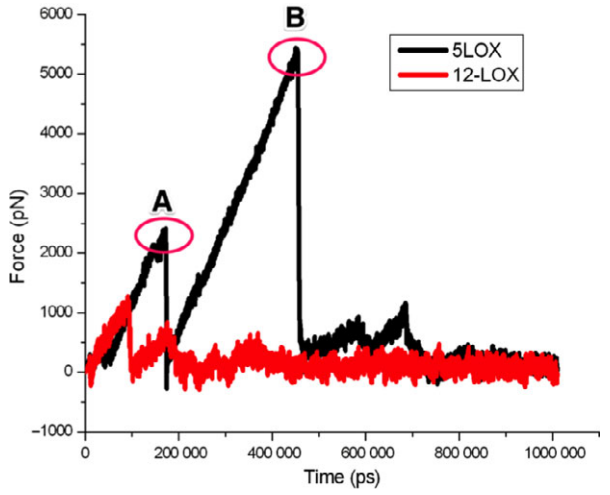


Figure 2: Variation of the pulling force (in pN) exerted on IR-2 during its unbinding from the 5-hLOX (black) and 12-pLOX (red) pockets.

a catechol group for potent inhibitory activity (12,13). To understand the inhibitory behavior of non-catechol compounds more thoroughly, additional evidence would be required; however, we can highlight that in our previous studies, IR-203 and IR-406 were unable to inhibit other human lipo-oxygenases (13), indicating they are selective for 5-LOX. An explanation could be based on the larger size of the 5-LOX active site, allowing for additional interactions with our tested compounds.

Table 2: Qualitative results of pseudoperoxidase assays for two standard compounds and three top inhibitors of human 5-LOX

Compound	Redox
Zileuton	Yes
Setileuton	No
HIR-303	Yes
HIR-309	Yes
IR-8	No

The oxidation state of ring C was not correlated with the potency of the inhibitors studied, as seen with the isoflavone/isoflavan pairs: IR-3/HIR-3, IR-301/HIR-301, IR-303/HIR-303, and IR-309/HIR-309, all having similar potencies. The IR-1/HIR-1 pair was an exception, with an over 100-fold difference in potency which may indicate that a different docking mode for this pair. Nevertheless, considering that the majority of the derivatives showed no potency difference between the IR and HIR derivatives, it appears that for 5-LOX, the oxidation state of ring C is not a critical factor for inhibitor potency. This differs from our previous results, where we observed that the oxidative state of the isoflavonoid had a large impact on 12-LOX and 15-LOX-1 potency and selectivity (12,13). In comparison, the structure–activity relationship of the isoflavones and isoflavans against porcine 5-LOX (11) showed similar behavior with human 5-LOX. This low sensitivity to the conformational change between isoflavones and isoflavans for 5-LOX may be due to its inherently larger active site, compared to the other LOX isozymes. One of the above pairs, IR-303 (**gray**) and HIR-303 (**pink**), were docked to the human 5-LOX active site and little difference was found between them. The catechol group was positioned at a mean distance of 4.9 Å from iron for both inhibitors (Figure S1), explaining the similar experimental results.

Docking studies also show that in the case of the 6,7-dihydroxy-isoflavones, the 7-hydroxyl of the catechol is at a distance of 4 Å from the active site iron. When the 6,7-dihydroxyisoflavone is reduced to obtain the 6,7-dihydroxyisoflavan, which changes the conformation of ring C, the 7-hydroxyl group of the ring B catechol group is still positioned the same as for the 6,7-dihydroxyisoflavone (Figure S2). These poses support the inhibitor data where similar potencies between the isoflavones and isoflavans were seen (i.e. IR-3, IC_{50} = 0.36 μM (**gray**) and HIR-3, IC_{50} = 0.28 μM (**pink**)).

The effect on 5-LOX inhibition by *para* or *meta* substituents on ring B is harder to interpret than the impact of the catechol position, previously described for ring A. The steric effects are not sufficient to explain the results. For example, IR-2 (IC_{50} 1.2 μM) had a 4′-methoxy group which is bigger than the 4′-hydroxyl on IR-1 (IC_{50} > 40 μM), but its potency is greater. The electronegativity of the substituents on ring B also does not explain the results obtained,

because we found a compound with an electron withdrawing nitro group (IR-6, IC_{50} 1.5 μM) and a compound with an electron donating methoxy group (IR-2, (IC_{50} 1.2 μM) both being good inhibitors. This observation was previously reported in our studies of the isoforms 12-LOX and 15-LOX-1 (12,13). The lack of importance of the ring B substituents for the inhibitory activity is reinforced with the results found for the following compounds: 7,8-IR (IR-301,-303,-308,-309), 6,7-HIR (HIR-1,-3,-7,-9), and 7,8-HIR (HIR-301,-303,-309). For all the above compounds, changes in size, electronegativity and position on ring B produced only minimal differences in their ability to inhibit 5-LOX.

Given the importance of the catechol moiety, it is logical to assume that these isoflavonoids are chelative and/or reductive inhibitors. To determine whether a particular inhibitor is chelative in nature, the EPR technique is required to observe a direct change in the iron ligation. This is a difficult experiment to do with 5-LOX, given the unstable nature of the enzyme (32,33). However, testing the reductive nature of an inhibitor is markedly easier with the pseudoperoxidase assay, which measures the reduction of the hydroperoxide product by 5-LOX, with the concomitant oxidation of the inhibitor. This test was performed with three potent 5-LOX inhibitors (IR-8, HIR-303, and HIR-309) and two known inhibitors, the chelative and reductive zileuton and the non-reductive setileuton (34). The results showed that both, HIR-303 and HIR-309, with 7,8-dihydroxy groups, tested positive in the pseudoperoxidase assay, which indicates that they are reductive inhibitors, most likely through an inner sphere mechanism that requires iron chelation. The radical scavenger properties presented by phenolic compounds with one or two hydroxyl groups were previously studied, especially for the catechol flavonoids taxifolin, luteolin, and quercetin, which can react with the metal ion producing ortho-benzoquinones by oxidation (35). However, IR-8, an isoflavone with a 6,7 dihydroxy group, was not active in the pseudoperoxidase assay, indicating that it is not a reductive inhibitor (Table 2). An explanation for this behavior might rely on the interruption of a fully conjugated system in isoflavans, and the important role of the acidity of the -OH group located at C-7 on ring A, as can be observed by analyzing the pK_a values for isoflavones and isoflavans, which are 6.7 and 9, respectively. This change in pK_a could be responsible for the different reductive properties of the inhibitors, allowing HIR derivatives to act as reductive inhibitors in the pseudoperoxidase assay, but not IR derivatives (12).

To display the position of these three inhibitors inside the binding site of 5-LOX, docking studies were performed. The data reveal that the catechol group of IR-8 was located symmetrically in front of the metal (see Figure 1A). On the other hand, the isoflavans, HIR-303 and HIR-309, were unable to orient their catechol groups symmetrically opposite to the metal, and only the 7-OH group was located near the iron atom. Nevertheless, these two metal chelating properties correlate well with a possible hydrogen transfer and change in oxidation state from ferric to ferrous iron supporting the pseudoperoxidase activity. (Figure 1B shows the docking of HIR-309, as an example.)

Table 3: Evaluation of inhibitory activity (IC_{50}) against COX1 and COX2 and selectivity of some of the better inhibitors of human 5-LOX

Compound (10 μM in assay)	COX1 IC_{50} [μM ± SD]	COX2 IC_{50} [μM ± SD]
Setileuton	>150	>100
Zileuton	>150	–
Ibuprofen	4.0 ± 0.4	8 ± 2
Aspirin	100%	19 ± 3
Baicalein	>150	–
IR-2	>150	>150
IR-8	>50	>150
IR-301	>150	>150
HIR-303	>150	>150
HIR-309	>150	>150

Twelve of the 26 isoflavonoid derivatives were screened against 15-LOX-2, and none showed potent activity (Table 1). Only HIR-9 had an IC_{50} value below 10 μM, which is over 10-fold less potent than its IC_{50} value against 5-LOX (IC_{50} = 0.5 μM). This lack of potency against 15-LOX-2 is a common feature for isoflavones and has been described previously (13). Nevertheless, HIR-9 is as potent (IC_{50} 8.3 μM) as NDGA (IC_{50} = 11 μM) (12), one of the few compound which inhibit 15-LOX-2 at all (36).

An additional aspect of this study was to evaluate whether some of the potent and selective 5-LOX inhibitors were also active against mammalian cyclooxygenase (COX), an enzyme also involved in arachidonic acid metabolism. The COX isozymes results showed that isoflavans HIR-303 and HIR-309 and isoflavone IR-2 do not inhibit COX-1 or COX-2 at IC_{50} concentrations higher than 150 μM (Table 3). Ibuprofen and aspirin were used as positive controls and shown to inhibit COX-2 activity with IC_{50} < 20 μM.

Among all the compounds tested, IR-2 was the most selective against 5-LOX (Table 1 and 2). Simulations by SMD studies showed difference of affinity when we analyzed their trajectory in both sites of 5 and 12-LOX (IC_{50} 1.2 and 100 μM, respectively). The SMD results in 5-LOX showed two significant maximum forces at 2500 and 5500 pN, the first peak (A) corresponds to the break of the interaction between catechol and iron inside to the binding site and the second peak corresponding to the rupture of the interaction between catechol and Pro152 and Leu153 residues (B). Finally, the SMD study for 12-LOX did not provide relevant results in affinity, and the only important signal was caused by the break of interaction between the catechol and the iron at 1300 pN. (Figure 2).

Table 4: *Ex vivo* inhibition of LTB_4 production (IC_{50}) in whole blood

Compound	IC_{50} [μM ± SD]
IR-8	19.4 ± 4.0
HIR-303	1.4 ± 1.2
HIR-309	1.2 ± 0.1
Setileuton	0.2 ± 0.1

The next step was to apply these inhibitors to a whole blood, 5-LOX cellular model, which would demonstrate not only cell permeability but also cellular stability. As seen in Table 4, HIR-303, HIR-309, and IR-8 (*in vitro* IC_{50} values of 0.18, 0.15 and 0.39 μM, respectively) all inhibit LTB_4 production *in cellulo*, indicating 5-LOX inhibition. However, the *in cellulo* IC_{50} values for HIR-303 (IC_{50} 1.4 μM) and HIR-309 (IC_{50} 1.2 μM) are approximately 10-fold higher than their *in vitro* IC_{50} values, with IR-8 being almost 50-fold less potent (IC_{50} 19.4 μM). These data indicate that while these compounds are active in the cell against 5-LOX, they have lowered potency, possibly due to either lowered cell permeability or structural modification of the compounds in the cell. It should be noted that the known 5-LOX inhibitor, setileuton, was used as a positive control and showed high activity.

Conclusion

A series of isoflavones and isoflavans were synthesized and evaluated against human leukocyte 5-LOX, with most of the compounds that possess a catechol moiety manifesting submicromolar potency, regardless of its position (i.e. 6,7- versus 7,8-). We also observe that substituents on ring B and ring C oxidation are not relevant for potency, but ring C oxidation is relevant for selectivity. None of these molecules are potent against the COX isozymes, and only one, HIR-9, shows modest potency against 15-LOX-2. The pseudoperoxidase assay shows that HIR-303 and HIR-309 are reductive inhibitors, but IR-8 is not, possibly due to an interruption in the fully conjugated system and its effect of -OH group acidity in HIR-303 and HIR-309, also both compounds are active against 5-LOX in whole blood, indicating effective cellular transport and cellular potency. In summary, we have demonstrated that both isoflavones and isoflavans can be potent and selective inhibitors against human leukocyte 5-LOX *in vitro* and *in cellulo*.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Scheme S1. Reagents and general conditions of classic synthesis of isoflavones and isoflavans.

Figure S1. Docking of 7,8-dihydroxyisoflavone and isoflavan derivatives inside the 5-LOX binding site (IR-303 and HIR-303, respectively).

Figure S2. Docking results of 6,7-dihydroxyisoflavone and 6,7-dihydroxyisoflavan derivatives IR-3 and HIR-3, respectively, inside the 5-LOX binding site.

Data S1. The isoflavones were characterized by standard characterization.