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Baicalein is a Potent In Vitro Inhibitor against both Reticulocyte 15-Human and Platelet 12-Human Lipoxygenase

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RECEIVED DATE (to be automatically inserted after your manuscript is accepted)

Running Title: Baicalein Inhibition of 12-hLO and 15-hLO

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Abstract. Lipoxygenases (LO) have been implicated in asthma, immune disorders, and various cancers and as a consequence, there is great interest in isolating selective LO isozyme inhibitors. Currently, there is much use of baicalein as a selective human platelet 12-LO (12-hLO) inhibitor however, our current steady-state inhibition data indicates that baicalein is not selective against 12-hLO versus human reticulocyte 15-LO-1 (15-hLO-1) (15/12 = 1.3), *in vitro*. However, in the presence of detergents baicalein is slightly more selective (15/12 = 7), which may imply greater selectivity in a cell based assay but has yet to be proven. The mechanism of baicalein inhibition of 15-hLO is reductive, which computer docking suggests is through direct binding of the catecholic moiety of baicalein to the iron. A structurally related flavonoid, apigenin, is not reductive, however, computer docking suggests a hydrogen bond with Thr591, may account for its inhibitor potency.

Keywords: lipoxygenase, baicalein, apigenin, flavonoids, kinetics, reductive inhibition, IC_{50}

Introduction

Lipoxygenases (LOs) are non-heme, iron-containing enzymes found in both the plant and animal kingdoms. LOs catalyze the dioxygenation of 1,4 cis,cis-pentadiene-containing polyunsaturated fatty acids (e.g., linoleic acid (LA) and arachidonic acid (AA)) to form hydroperoxy-fatty acids (Scheme 1).[1] In mammals, this is the first step in the biosynthesis of leukotrienes and lipoxins, which are critical biological, signaling molecules.[2, 3] There are three major human LOs (hLOs), 5-, 12-, and 15-hLO, whose main difference is the position of dioxygen incorporation into arachidonic acid (AA) (C-5, C-12, or C-15).[4, 5] These three hLO isozymes are of great interest to scientists because they have been shown to be involved in a variety of diseases; 5-hLO in prostate cancer[6, 7] and asthma[8], 12-hLO in immune disorders[9] and breast cancer[7, 10, 11], and 15-hLO-1 in atherosclerosis[12] and colorectal cancer.[7, 13] Due to their involvement in such diseases, a better understanding of the mode of inhibition of small molecules is necessary to aid in future rational drug design against specific LO isozymes.

In the literature, there are numerous reports of 5-hLO and 15-hLO-1 specific inhibitors but less so for platelet 12-hLO. For this reason and its well documented role in cancer progression, our laboratory has become increasingly interested in discovering selective platelet 12-hLO inhibitors. [14-20] Plant extracts are a rich source of LO inhibitors [16, 21-23] and one particular class, the flavonoids, is relatively nontoxic, phenolic, and potent against LO.[16, 24-27] In addition, flavonoids have been shown to have antioxidant[24, 28], anti-inflammatory[26, 29], antitumor[30], antimicrobial[31] and antiviral properties[32]. One specific flavonoid LO inhibitor is baicalein, a major component in the root of *Scutellaria baicalensis* (1.9% of total root), and has been shown to induce apoptosis in breast, prostate, colon and pancreatic cancer cell lines.[33-36] In all cases, the potency of baicalein is thought to be due to the selective inhibition of platelet 12-hLO,[37-43] thus interrupting only part of the arachidonic acid metabolic pathway, however, the basis of this supposition is unclear. The most cited reference, by Sekiya and Okuda, indicated only that baicalein was selective to platelet 12-hLO versus cyclooxygenase (COX), but not versus the other LO isozymes.[44] In order to investigate this discrepancy in the literature, we performed extensive steady-state inhibition kinetics with baicalein and a flavonoid homologue, apigenin, in order to assess their selectivity against platelet

12-hLO and reticulocyte 15-hLO-1 *in vitro* and whether experimental conditions, such as detergents could effect their inhibitor potency (Figure 1).

Insert Scheme 1 and Figure 1

Results

Expression and Purification of Lipoxygenases. Human platelet 12-LO (12-hLO) and human reticulocyte 15-LO-1 (15-hLO-1) were purified with yields of ≈ 50 mg/L of SF9 insect cells. ICP-MS data indicated that 12-hLO had 12 ± 1% iron content and 15-hLO-1 had 24 ± 2% iron content. All kinetic data was adjusted for iron content.

Baicalein Steady-State Inhibition Kinetics Studies of 12- and 15-hLO-1. The observed steady-state rate of catalysis was determined by measuring the formation of 12-HPETE or 15-HPETE as a function of enzyme concentration, substrate concentration, and inhibitor concentration. K_m and K_{cat} values were obtained from Michaelis-Menton fits, while K_i and K_i' values were determined by applying standard kinetic equations.[45] For 12-hLO, plots of slopes and y-intercepts versus baicalein are shown in Figure 2A and 2B and represent linear mixed inhibition. The plots are linear and give two different -K_i values at the x-intercepts. The x-intercept calculated from the slope versus [I] plot represents K_i (0.14 ± 0.11 μM), and the x-intercept of the y-intercept versus [I] plot represents the K_i' (3.1 ± 0.27 μM) (Table 1).[45] This is considered linear mixed inhibition where the two different equilibrium constants for inhibitor dissociation, K_i and K_i' are defined as the equilibrium constant of the dissociation of inhibitor from the catalytic site and a secondary site, possibly an allosteric binding site[46], respectively.

Insert Figure 2 and Table 1

For 15-hLO-1, baicalein showed competitive inhibition under non-detergent buffer conditions (25 mM Hepes buffer, pH 7.5) The plots of the slopes and the K_m values versus baicalein concentration for 15-hLO-1 are shown in Figure 3A and 3B, respectively. Both plots yield linear graphs where -K_i is the x-intercept. Both K_i values (0.22 ± 0.04 μM for figure 3A and 0.14 ± 0.05 μM for figure 3B) are within error of each other indicating competitive inhibition.[45] The average of the equilibrium inhibitor constants gives a K_i of 0.18 ± 0.05 μM (Table 1). The steady-state inhibitor kinetics were also performed in the

presence of 0.01% triton-X-100, which changed the inhibitor response of 15-hLO-1. In the presence of triton, baicalein showed linear mixed inhibition towards 15-hLO-1, similar to that with 12-hLO, with a K_i equal to 1.01 ± .05 μM and a K_i' equal to 14.25 ± 1.25 μM (Table 1).

Insert Figure 3

Apigenin Steady-State Inhibition Kinetics Studies of 12-hLO and 15-hLO-1. For 12-hLO, apigenin showed linear mixed inhibition. The dissociation equilibrium constants, K_i and K_i', were determined as previously described for baicalein, with a K_i equal to 14 ± 7.4 μM and a K_i' equal to 120 ± 2.8 μM.[45] For 15-hLO-1, apigenin demonstrated competitive inhibition, with an average K_i of 2.0 ± 1.0 μM (Table 1).

IC_{50} Analysis. IC_{50} studies of both 12- and 15-hLO-1 were performed as previously described (plots not shown).[15] Without 0.01% triton-X-100 in the buffer, baicalein had an IC_{50} of 0.64 ± 0.11μM against 12-hLO and 1.6 ± 0.24 μM against 15-hLO-1. Apigenin had an IC_{50} of 81 ± 32 μM against 12-hLO and 3.4 ± 0.51μM against 15-hLO-1 (Table 2). With 0.01% trition-X-100 in the buffer, baicalein had an IC_{50} of 0.62 ± 0.19μM against 12-hLO and 38 ± 21 μM against 15-hLO-1. Apigenin had an IC_{50} of 32 ± 11 μM against 12-hLO and 3.0 ± 1.4 μM against 15-hLO-1 (Table 3).

Insert Tables 2 and 3

Pseudoperoxidase Assay. Pseudoperoxidase studies of 15-hLO-1 were performed as previously described to determine if a particular inhibitor could function as a reductant to the active site iron.[47] Baicalein's mode of inhibition against 15-hLO-1 proved to follow a redox mechanism as seen previously with NDGA and other catechol like compounds,[19] while apigenin followed reversible binding inhibition. It should be noted that the pseudoperoxidase activity was reliable and consistent with 15-hLO-1, but for 12-hLO, only the inhibitor BWB70C could support the assay. This difference maybe due to the fact that the pseudoperoxidase assay detects only a small percentage of hydroperoxide decomposition (loss of 234 nm), which could be less probable for the 12-hLO reaction with arachidonic acid, and requires further investigation.

Molecular Modeling Analysis. The three protonation states of baicalein were each flexibly docked into the active site of the 15-hLO-1 model.[48] Considering the proximity of the iron atom to baicalein and the electron withdrawing hydroxides on baicalein, the assumption of a singularly deprotonated baicalein at pH 7.5 is reasonable. All three forms of baicalein docked to the active site produced poses with the 6-carbon phenoxide pointing towards the iron at distances ranging from 2.6 – 3.5Å (Figure 4A, only the top pose shown for clarity). The two de-protonation states of apigenin also were docked into the active site of the 15-hLO-1 model and found to dock in a different manner than baicalein. The result of the docking simulation resulted in multiple possible molecular interactions of apigenin and 15-hLO-1, however, in no instance was a phenolate group on apigenin found to approach the iron atom closer than 4.0Å (Figure 4B, only the top pose shown for clarity).

Insert Figure 4

Discussion

Our laboratory has investigated lipoxygenase inhibitors from many sources with the goal of identifying compounds with both unique chemical scaffolds and selectivity against specific LO isozymes. To date, we have characterized a number of inhibitors from both marine sponges and plants, however their selectivity is predominantly against reticulocyte 15-hLO-1 and not platelet 12-hLO.[14-18] This fact has inspired us to search further for 12-hLO selective inhibitors due to its well documented role in various human diseases.[7, 9-11]

In the current paper, we investigated the flavonoids, baicalein and apigenin, as possible 12-hLO selective inhibitors, because, baicalein has been used in numerous citations as a selective inhibitor against 12-hLO in mammalian cells.[37-43] Nevertheless, our IC_{50} data showed that there was minimal selectivity between 12-hLO and 15-hLO-1 with baicalein *in vitro* (15/12 = 2.5). Due to this discrepancy between our data and the presumptions in the literature, we decided to perform extensive steady-state kinetics to confirm our results. The steady-state kinetics data corroborated our IC_{50} data, confirming that baicalein is not selective against 12-hLO *in vitro* (15/12 = 1.3). We then performed IC_{50} experiments with both 12- and 15-hLO-1 in the presence of triton-X-100 to determine if inhibitor aggregation was a

mitigating factor. In 2003 Ryan et al. showed that in the absence of detergent, some compounds tend to form aggregates.[49] These aggregates, termed "phony" inhibitors, are proposed to inhibit by non-specific absorption onto the surface of enzymes and are not considered suitable as possible drug leads. Our IC_{50} data indicated that detergent had no effect on baicalein inhibition of 12-hLO, but it did have an effect on 15-hLO-1 inhibition, increasing its selectivity (15/12 = 58). We therefore extended our study and performed the more accurate steady-state inhibition kinetics with 15-hLO-1 and baicalein in the presence of detergent and determined that the K_i of baicalein against 15-hLO-1 increased with detergent present but less than the IC_{50} data had suggested. The steady state inhibition data indicates that the inhibitor selectivity (15/12) at the catalytic site (K_i) is only 7, markedly lower than the 58 seen with the IC_{50} data. Considering that the K_i for 15-hLO-1 is low (1 uM with detergent), we consider this mild selectivity at best. It should be noted that the fact that baicalein inhibition of 15-hLO-1 was affected by detergent but the inhibition of 12-hLO was not, is unusual because the buffer conditions are nearly identical between the two enzyme assays and it is unlikely that baicalein only aggregates under the 15-hLO-1 assay conditions and is soluble under the 12-hLO assay conditions.

In order to investigate this detergent effect further, we included another flavonoid, apigenin, which we previously demonstrated to be a potent LO inhibitor.[16] Apigenin is a good candidate for comparison with baicalein due to its similar structure to baicalein, except for the re-positioning of one alcohol group (Figure 1). The steady-state kinetics data indicated that apigenin is a linear mixed inhibitor against 12-hLO (K_i = 14 and K_i' = 120 uM), while it is a competitive inhibitor against 15-hLO-1 (K_i = 2 uM). The addition of detergent to the assay buffer had minimal effect, on the IC_{50} inhibition values of either 12-hLO or 15-hLO-1. This lack of detergent dependency of apigenin inhibition is consistent with the hypothesis that apigenin does not form inhibitor aggregates, while baicalein does. Nevertheless, this hypothesis seems unlikely due to the similar structure between baicalein and apigenin, and the fact that their low cLogP values of 3 indicate high solubility for both compounds in water. An alternative explanation could be that detergents change the overall structure of 15-hLO-1 in such a way that the potency of baicalein is lowered but not that of apigenin. Given the fact that lipoxygenases are known to

associate with the lipid bilayer, a structural change upon addition of detergent is feasible, however, further studies are needed to clarify this unusual detergent effect.

With regards to the nature of the baicalein inhibitory mechanism, we assumed that baicalein was a reductive inhibitor due to its catecholic scaffold. Numerous other catechol inhibitors are reductive inhibitors[19] but it had never been directly demonstrated whether baicalein was a reductive inhibitor against human LO or not. Utilizing the established psuedoperoxidase assay[47], we show that baicalein is a reductive inhibitor against 15-hLO-1. Based on our knowledge of baicalein, it is reasonable to assume that a catecholic alcohol ligates the iron and causes an inner sphere reduction on the active site iron, with baicalein undergoing oxidation to its quinone form (Figure 5).[50] Apigenin was also investigated for pseudoperoxidase activity, however, it is not active, indicating apigenin is not a reductive inhibitor. This difference between baicalein and apigenin could be due to their different structures and chelation properties. Baicalein can chelate the iron and reduce it, while apigenin cannot, because it does not contain a catechol moiety. We therefore docked both baicalein and apigenin into a 15-hLO-1 structural model and determined that baicalein can bind to the iron via its 6-carbon alcoholic moiety and hence perform an inner sphere reduction of the iron. The poses demonstrate the ability of the baicalein molecule to approach the iron for chelation (distance to iron = 2.6Å), whereas the apigenin molecule remains too far from the iron for a reductive inhibitory mechanism to occur (distance to iron = 4.0Å)(Figure 4A and 4B). Nevertheless, apigenin's ability to inhibit LO may be due to a hydrogen bond between its terminal alcohol group and residue T591 (1.8Å), which may help anchor apigenin in an orientation that blocks substrate accessibility to the iron.

Insert Figure 5

In summary, this investigation demonstrates that for human lipoxygenases, IC_{50} values only provide an approximate measure of inhibitor potency. They tend to manifest higher inhibitor values than the steady-state K_i value due to the fact that if the inhibitor is non-competitive, the IC_{50} value becomes an average between K_i and K_i' and therefore care should be taken in their analysis. Second, baicalein inhibition of 15-hLO-1 is sensitive to detergent concentrations while 12-hLO is not. This could be due

to either inhibition aggregation or detergent interaction with 15-hLO-1 and requires further study. Third, our data confirms that baicalein is a redox inhibitor against 15-hLO-1, which most likely binds directly to the catalytic iron through its catechol moiety, while apigenin does not. Finally, and most importantly, baicalein is not selective against platelet 12-hLO in the absence of detergents (15/12 = 1.3) and is only slightly selective in the presence of detergents (15/12 = 7), *in vitro*, which raises the question of how selective baicalein is in a cell based assay. Considering the extensive use of baicalein in cellular systems to date as a 12-selective inhibitor,[37-43] our data indicates that it is imperative to show if baicalein is 12-hLO selective in cell culture and animal models.

Experimental

Materials. Arachidonic acid (AA), Linoleic acid (LA), baicalein and apigenin were purchased from Sigma-Aldrich Chemical Company. All other reagents were reagent grade or better and were used without further purification.

Reverse Phase-HPLC Purification of AA and LA. AA and LA were purified as published,[51] using a Higgins Preparative Haisil (250 × 10mm) C-18 5uM column. An isocratic elution of 85% A and 15% B (Solvent A: 99.9% MeOH 0.1% acetic acid, Solvent B: 99.9% H2O and 0.1% acetic acid) was used to purify the fatty acids and both were stored in 95% EtOH at -20°C.

Expression and Purification of Lipoxygenases. Human platelet 12-lipoxygenase (12-hLO) and reticulocyte 15-lipoxygenase (15-hLO-1) are N-terminus, His_6-tagged proteins and were expressed/purified as described previously.[18] Iron contents of both lipoxygenase enzymes were determined using a Finnegan inductively coupled plasma mass spectrometer (ICP-MS), using cobalt-EDTA as an internal standard. LO iron concentrations were compared to standardized iron solutions.

Steady-State Inhibition Kinetics Studies. Lipoxygenase rates were determined by following the formation of the conjugated diene product at 234 nm (ε = 25000 M-1 cm-1) with a Perkin-Elmer Lambda 40 UV/Vis spectrophotometer. All reactions were 2 mL in volume and constantly stirred using a magnetic stir bar at room temperature (23ºC). Reactions with 12-hLO were carried out in 25 mM Hepes buffer (pH 8) in the presence of AA. Reactions with 15-hLO-1 were carried out either in 25 mM

Hepes buffer (pH 7.5) in the presence of LA or under the same conditions with 0.01% triton-X-100 added. AA and LA concentrations were quantitatively determined by allowing the enzymatic reaction to go to completion. Michaelis-Menton kinetics were determined for 12-hLO and 15-hLO-1 with their respective substrates and at varying inhibitor concentrations, from 0.38 to 80 μM. Enzymatic reactions were initiated by the addition of ≈ 5 nM 12-hLO and ≈ 9 nM 15-hLO-1. Kinetic data were obtained by recording initial enzymatic rates at each substrate concentration and then fitting them to the Michaelis-Menton equation using the KaleidaGraph (Synergy) program. All inhibitors were studied in separate experiments against each enzyme at least three times to determine their mode of inhibition. Inhibitor binding constants (K_i and K_i') were determined as described previously.[45] All other kinetic data were analyzed in a similar way.

IC_{50} Assay. Lipoxygenase rates were determined using the same method as previously described in the steady-state section, but with a Hewlett-Packard 8453 UV/Vis spectrophotometer. All reactions were 2 mL in volume, constantly stirred using a magnetic stir bar at room temperature (23°C) (with and without 0.01% triton-X-100), ≈ 9 nM of both enzymes and with 2.5 μM substrate. IC_{50} values were obtained by determining the enzymatic rate at various inhibitor concentrations then plotting them against inhibitor concentration. The data was fit to a saturation curve and the inhibitor concentration at 50% activity was determined (IC_{50}). Inhibitors were stored at -20°C in MeOH or DMSO depending on their solubility.

Pseudoperoxidase Activity Assay. Pseudoperoxidase activity of both 12-hLO and 15-hLO-1 was determined as previously described.[47] Pseudoperoxidase activity was monitored by following the degradation of 13(*S*)-hydroperoxyoctadecadieneoic acid (13-HPOD) at 234 nm. All reactions were performed in 2mL of buffer at room temperature (23°C), with a known lipoxygenase redox inhibitor BWB70C as the control.[52, 53]

Molecular Modeling Studies. The 15-hLO-1 homology model was created using the Protein Local Optimization Program (PLOP, commercially distributed as Prime), which uses loop prediction[54], side chain prediction[55, 56] and energy minimization to align the target and template sequences, as previously

reported.[48] The structures of apigenin and baicalein was prepared for docking using the LigPrep (Schrödinger, Inc) ligand preparation software, which generates a minimized conformation of each ligand, and multiple protonation/tautomerization states when appropriate. Flexible ligand docking was performed using the Glide (Schrödinger, Inc.) program,[57, 58] which uses a modified version of the Chemscore energy function to score the protein-ligand interactions.[59]

Acknowledgment. Financial support was from the National Institute of Health grant GM 56062-06 and the American Cancer Society grant RPG-00-219-01-CDD.

Scheme 1. Lipoxygenase reaction

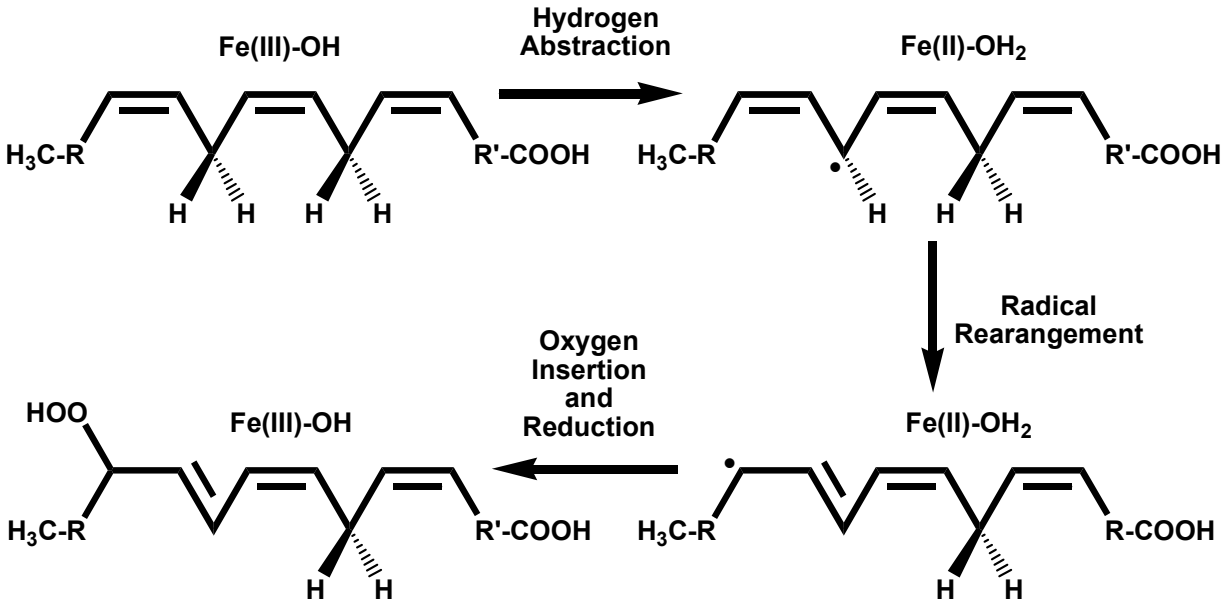


Table 1. Steady-state inhibition data for 12- and 15-hLO-1 with their respective inhibitors.

`	**Baicalein**	**Apigenin**
12-hLO	K_i = 0.14 ± 0.11 μM $K_i^{'}$ = 3.1 ± 0.27 μM	K_i = 14 ± 7.4 μM $K_i^{'}$ = 120 ± 2.8 μM
15-hLO-1	K_i = 0.18 ± 0.05 μM	K_i = 2.0 ± 1.0 μM
15-hLO-1 w/.01% triton-X-100	K_i = 1.01 ± 0.05 μM $K_i^{'}$ = 14.25 ± 1.25 μM	

Table 2. IC_{50} data (without 0.01% trition-X-100) for 12- and 15-hLO-1 with their respective inhibitors.

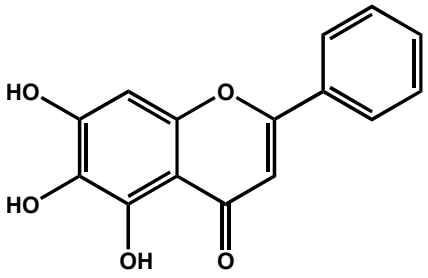
	Baicalein	**Apigenin**
12-hLO	IC_{50} = 0.64 ± 0.11 μM	IC_{50} = 81 ± 32 μM
15-hLO-1	IC_{50} = 1.6 ± 0.24 μM	IC_{50} = 3.4 ± 0.51 μM

Table 3. IC_{50} data (with 0.01% trition-X-100) for 12- and 15-hLO-1 with their respective inhibitors.

	Baicalein	**Apigenin**
12-hLO	IC_{50} = 0.63 ± 0.19 μM	IC_{50} = 33 ± 11 μM
15-hLO-1	IC_{50} = 37 ± 1.5 μM	IC_{50} = 3.0 ± 1.4 μM

Figure 1. Structures of baicalein (**A**) and apigenin (**B**).

A



B

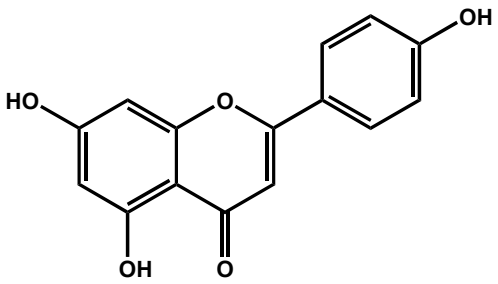


Figure 2. Linear mixed inhibition steady-state kinetics data for determination K_i and $K_i^{'}$ for 12-hLO with Baicalein. Figure 2A, Slope vs. [Baicalein] μM is the secondary re-plot of inhibition data used to get K_i. Figure 2B, y-intercept vs. [Baicalein] μM is also a secondary re-plot of inhibition data used to get $K_i^{'}$.

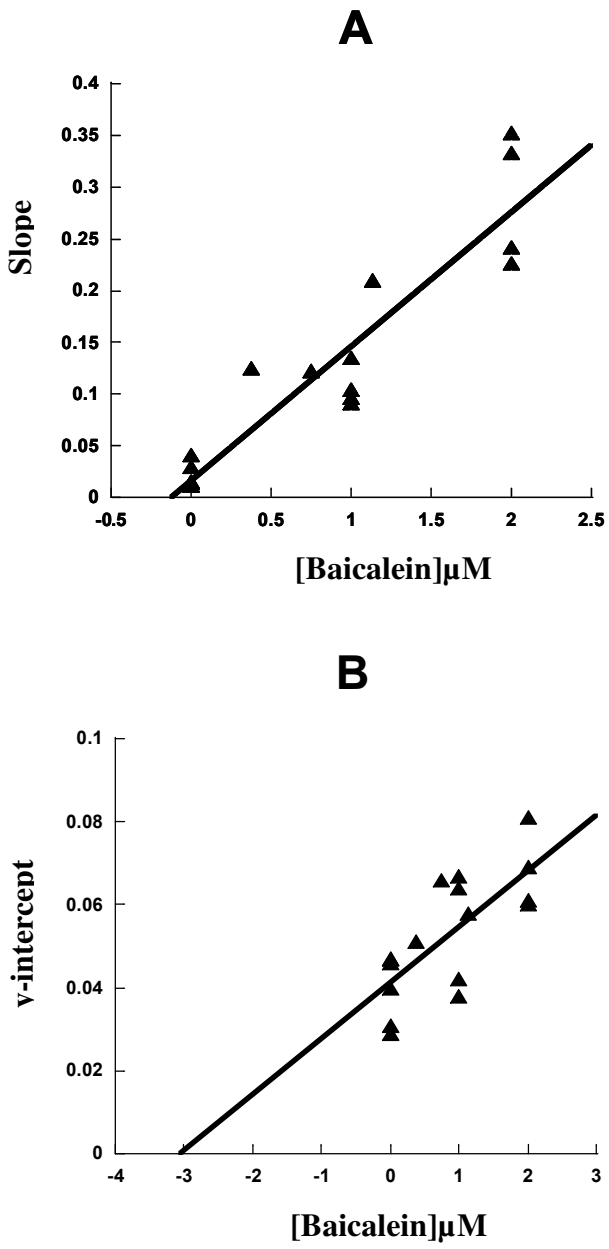


Figure 3. Competitive inhibition steady-state kinetics data for determination K_i for 15-hLO-1 with Baicalein. Figure 3A, Slope vs. [Baicalein] μM is the secondary re-plot of inhibition data used to get K_i. Figure 3B, K_m vs. [Baicalein] μM is also a secondary re-plot of inhibition data used to get K_i.

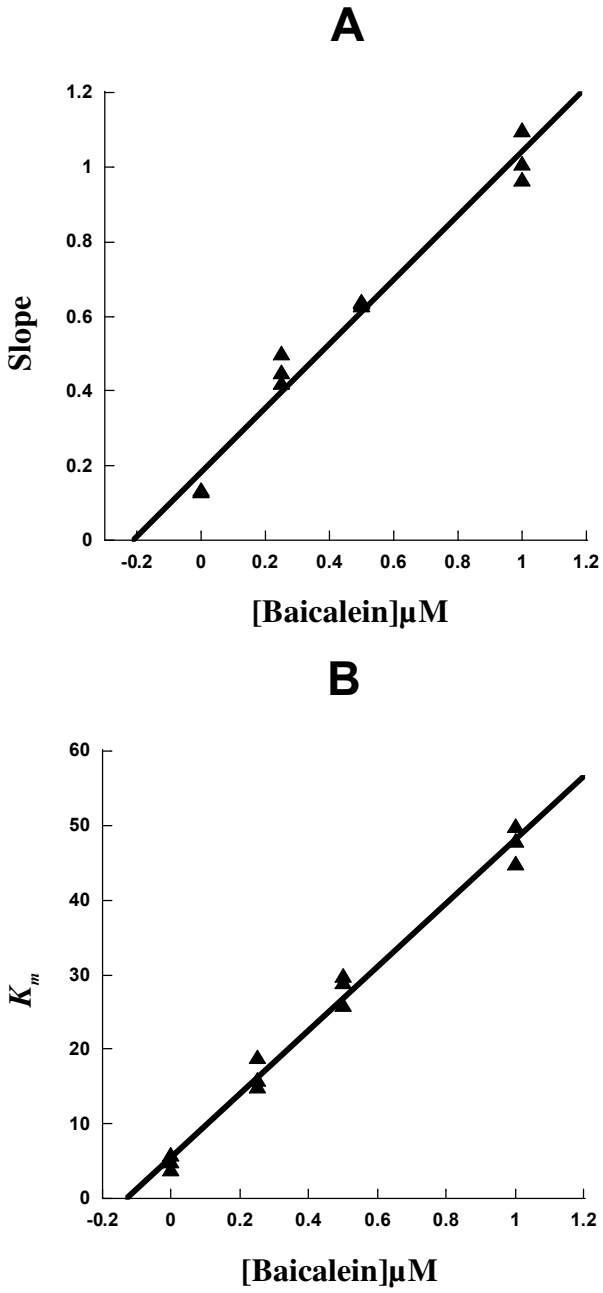


Figure 4. Representative poses of docking simulations for 15-hLO-1 with Baicalein (4A) and Apigenin (4B). The distances of baicalein and apigenin from the iron are 2.6Å and 4.0Å, respectively. Apigenin's distance from T591 is 1.8Å.

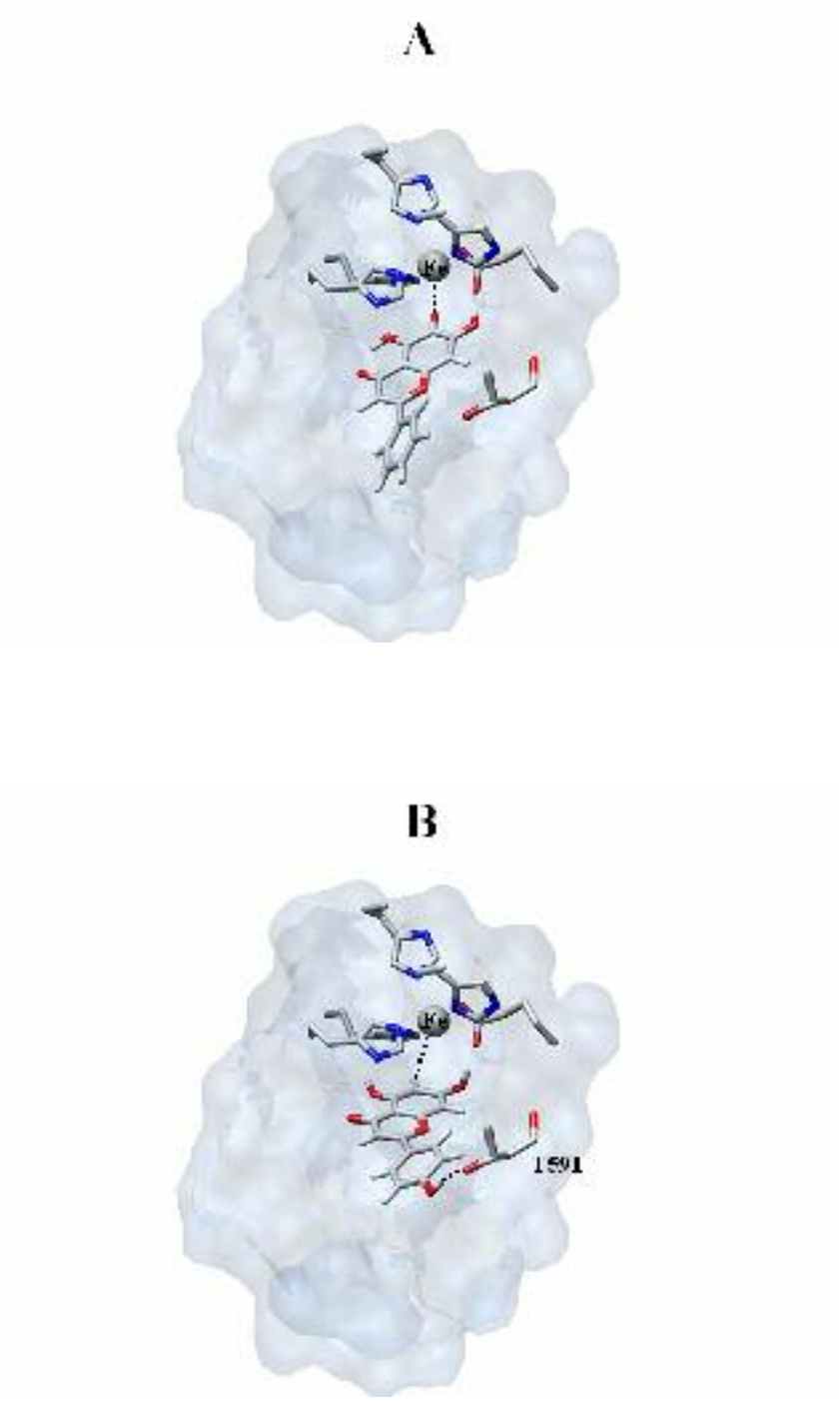
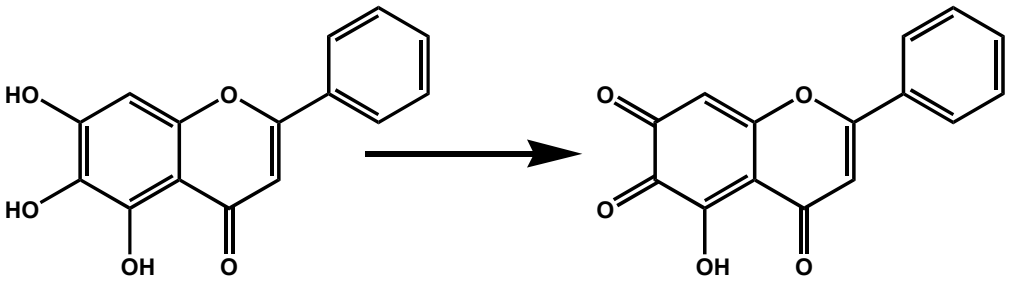
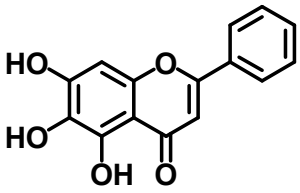
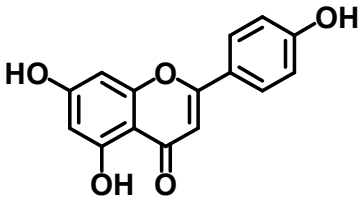


Figure 5. Redox mechanism of baicalein with both 12- and 15-hLO-1.



Baicalein **Baicalein quinone**

Table of Contents Graphic:

	Baicalein	**Apigenin**
		
12-hLO IC_{50} =	**K_i = 0.14 ± 0.11 µM** **K_i' = 3.1 ± 0.27 µM**	**K_i = 14 ± 7.4 µM** **K_i' = 120 ± 2.8 µM**
15-hLO IC_{50} =	**K_i = 0.18 ± 0.05 µM**	**K_i = 2.0 ± 1.0 µM**

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