Title
Synthesis And Structure-Activity Relationships Studies Of 4-((4-Hydroxy-3-Methoxybenzyl)Amino)Benzenesulfonamide Derivatives As Potent And Selective Inhibitors Of 12-Lipoxygenase

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SYNTHESIS AND STRUCTURE-ACTIVITY RELATIONSHIPS STUDIES OF
4-((4-HYDROXY-3-
METHOXYBENZYL)AMINO)BENZENESULFONAMIDE DERIVATIVES AS
POTENT AND SELECTIVE INHIBITORS OF 12-LIPOXYGENASE

A thesis submitted in partial satisfaction
of the requirements for the degree of

MASTER OF SCIENCE

In

CHEMISTRY

By

Auric Sowell-Kantz

March 2014

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Abstract

SYNTHESIS AND STRUCTURE-ACTIVITY RELATIONSHIPS STUDIES OF 4-((4-HYDROXY-3-METHOXYBENZYL)AMINO)BENZENESULFONAMIDE DERIVATIES AS POTENT AND SELECTIVE

Auric Sowell-Kantz

Human lipoxygenases (LOXs) are a family of iron-containing enzymes which catalyze the oxidation of polyunsaturated fatty acids to provide the corresponding bioactive hydroxyeicosatetraenoic acid (HETE) metabolites. These eicosanoid signaling molecules are involved in a number of physiologic responses such as platelet aggregation, inflammation, and cell proliferation. Our group has taken a particular interest in platelet-type 12-(S)-LOX (12-LOX) because of its demonstrated role in skin diseases, diabetes, platelet hemostasis, thrombosis, and cancer. We previously reported the discovery of ML127, a potent and selective 12-LOX inhibitor which has proven to be a valuable tool compound for researchers in the field. However, the limited tolerance for structural modification and modest PK profile for this chemotype prompted us to continue our discovery efforts toward novel 12-LOX inhibitors. Herein, we report the identification and medicinal chemistry optimization of a 4-((2-hydroxy-3-methoxybenzyl)amino)benzenesulfonamide-based scaffold. Top compounds, exemplified by 35 (ML355) and 36, display nM potency against 12-LOX and excellent selectivity over related lipoxygenases and cyclooxygenases. In addition to possessing favorable ADME properties, 35 and 36 inhibit PAR-4 induced aggregation and calcium mobilization in human platelets, and reduce 12-HETE in mouse/human beta cells.
INTRODUCTION:

Lipoxygenases are a class of non-heme iron-containing enzymes which regio- and stereospecifically oxidize polyunsaturated fatty acid substrates such as arachidonic acid (AA) and linoleic acid (LA). The position at which these cis, cis-1,4-pentadiene substrates are oxidized correspond to the requisite lipoxygenase, with the three major human lipoxygenases: 5-LOX, 12-LOX, and 15-LOX-1, oxidizing the C-5, C-12 and C-15 positions respectively. Lipoxygenases are involved in the first committed step in a cascade of metabolic pathways and the products of these enzymes (eicosanoids) are precursors of hormones such as leukotrienes and lipoxins, which mediate a wide array of cellular functions. Consequently, the lipoxygenase enzymes and their bioactive metabolites (e.g. hydroxyeicosatetraenoic acid (HETE) and leukotriene A4) have been implicated in a variety of inflammatory diseases and cancers. Specifically, 5-LOX has been implicated in cancer and inflammatory diseases such as asthma and remains the only lipoxygenase enzyme for which there is an FDA-approved inhibitor (Zilueton) on the market. Reticulocyte 15-LOX-1 has received particular attention for its role in atherogenesis, neurodegenerative diseases, and neuronal damage associated from an acute ischemic stroke event. 12-LOX exists as three isozymes, platelet-type, leukocyte, and epidermal, however leukocyte 12-LOX is not found in humans yet is present in rat, mouse, pig and cow. Platelet-type 12-(S)-LOX (12-LOX), which the current study focuses on, has been found to be overexpressed in a variety of tumor tissues including prostate cancer, colorectal cancer, breast cancer and lung cancer. Moreover, 12-HETE levels have been linked
to increased cancer cell metastasis by facilitating tumor cell motility and angiogenesis.  

12-LOX is also expressed in human pancreatic islets, which is upregulated and activated by inflammatory cytokines leading to increased 12-LOX translocation. The resulting 12-HETE product leads to reduced insulin secretion, reduced metabolic activity and pancreatic β cell death through the amplification of the inflammatory response. Toward this end, both non-obese diabetic (NOD) 12-LOX and 12-LOX KO mice showed significant resistance to the development of diabetes compared to the controls, suggesting 12-LOX is a clear regulator in this disease. 12-LOX and its product 12-HETE have also been implicated in the modulation of hemostasis and thrombosis via their role in regulating platelet function (reactivity, clot formation, calcium mobilization). Historically the precise role of 12-HETE has been controversial with reports suggesting both an anti- and pro-thrombotic effects. One difficulty in being able to clearly define the role of 12-LOX in these systems has been the lack of potent and selective 12-LOX small molecule inhibitors. However, working with the inhibitor, NCTT-956, which is similar to our previous series (ML127), we were able to show that inhibition of 12-LOX leads to reduced platelet aggregation and calcium mobilization following stimulation by various agonists (PAR1-AP, PAR4-AP, Collagen, thrombin). These results helped further validate 12-LOX as a potential target for anti-platelet therapy.

Our laboratories and others have been actively engaged for many years in the discovery of novel potent 12-LOX inhibitors utilizing traditional medicinal chemistry, computational chemistry and natural product isolation. However, despite these
extensive efforts, no drug-like small molecule that was either chemically tractable or
selective had been discovered. Instead, these efforts yielded compounds that were
either reductive and/or promiscuous in nature, as exemplified by the inhibitors listed
in Figure 1. The known reductive LOX inhibitor, nordihydroguaiaretic acid
(NDGA)\textsuperscript{xviia} and other natural products including the brominated aryl phenols\textsuperscript{xviic}
which are marine derived natural products, all have modest 12-LOX inhibitory
activity (micromolar). Importantly, these compounds lack selectivity towards 12-
LOX and have undesirable chemical structures. For example, the polyphenolic
compound, Baicalein, was once one of the most commonly used 12-LOX inhibitors
as it was originally thought to be selective for 12-LOX. However, subsequent studies
revealed equipotent \textit{in vitro} activity towards both 12-LOX and 15-LOX-1.\textsuperscript{xviib}

Given the previously stated biological importance of 12-LOX and the lack of high
quality probe compounds in the literature, we initiated a high throughput screening
campaign as part of the MLPCN program. This effort led to the discovery of an 8-
Hydroxyquinoline based compound (ML\textsubscript{127}) which exhibited excellent selectivity,
>50-100 fold selectivity over related lipoxygenases and cyclooxygenase. In contrast to
many of the previously reported inhibitors, kinetic experiments revealed that these
inhibitors were non-competitive and non-reductive. Chiral HPLC separation of the
probe molecule revealed a chiral preference for activity with the (−)-enantiomer being
much more potent than the (+)-enantiomer (<0.5 μM vs. >25 μM, respectively).\textsuperscript{xxi}
However, despite this promising activity the chemical series was difficult to optimize
further, given that subtle structural modifications led to diminished activity.
Therefore, we sought to reexamine the results from the original HTS to uncover additional compounds that may be amendable to medicinal chemistry optimization. Ultimately, this led to the identification of a 4-((2-hydroxy-3-methoxybenzyl)amino)benzenesulfonamide-based scaffold which was subjected to medicinal chemistry optimization and biological characterization. The results of these efforts are described herein.

![Figure 1](image1.png)

**Figure 1.** Representative examples of previously reported 12-LOX inhibitors

**CHEMISTRY:**

Prior to initiating an extensive SAR campaign around the HTS “hit” molecule, (1), the NIH first sought to resynthesize the molecule to confirm its identity and activity. The synthesis of compound 1 involved a reductive amination with 4-aminobenzesulfonamide and 2-hydroxy-3-methoxybezaldehyde. While this route appeared amendable to the facile preparation of numerous synthetic analogs, the standard mild method for reductive aminations utilizing sodium...
triacetoxyborohydride and catalytic acetic acid afforded little to no product. Instead, a step-wise approach involving pre-formation of the imine was required with 4-amino-N-(thiazol-2-yl)benzenesulfonamide (2) and the requisite benzaldehyde overnight in ethanol at reflux. Subsequent reduction of the imine with sodium borohydride provided the desired compounds 1, 8-10 and 14-33 (Scheme 1, Method A). While this route worked for a majority of the analogs, some analogs (11-13 & 34) required an alternative route in which a Buchwald-Hartwig type coupling was utilized with the commercially available 4-bromo-N-(thiazol-2-yl)benzenesulfonamide (3) and 2-methoxy substituted benzyl amines to provide the desired products in a yields of 25-40% (Scheme 1, Method B). For modifications of the thiazole portion of the molecule we wanted to introduce diversity in the final step of the synthesis (Scheme 1, Method C). Therefore, reaction of commercially available 4-aminobenzenesulfonamide (4) with 2-hydroxy-3-methoxybenzaldehyde in ethanol at reflux for 6 h, followed by treatment with sodium borohydride provided compound 5 in 95% yield. The resulting sulfonamide derivative was then subjected to Cu-catalyzed N-arylation conditions using the appropriate heteroaryl bromides to afford compounds 35, 38, 44-48, 52, 53, 56-59, 61-69, 71, 74, and 75 in good yields. Despite the general versatility of this method, there was a few isolated cases where the Cu-catalyzed reaction failed to produce the desired product (e.g. compounds 77-83), or the heteroaryl bromides were not readily available. For these compounds a less direct route was developed by heating 4-nitrobenzene-1-sulfonyl chloride (6) and the required heteroaryl amines to 100°C for 1.5 to 18 hours depending on the
reactivity of the amine to give the 4-nitrophenyl-sulfonamide derivatives 7 (Scheme 1, Method D). Reduction of the nitro group was achieved using the H-Cube® Pro flow reactor with 10% Pd on carbon at 50 °C and a pressure of 50 bar. Alternatively, for less soluble compounds which were not amenable to flow chemistry, a Zn/AcOH reduction was performed at 60 °C in methanol. Once the desired aniline was in hand a reductive amination was carried out with the corresponding benzyl amine derivative to provide compounds 36, 37, 39-43, 49-51, 54, 55, 60, 70, 72, 73, and 76-83.

Scheme 1. Synthesis of analogs 1-83.

A

\[
\begin{align*}
\text{R} \text{CHO} & \quad (1.5 \text{ equiv}), \text{EtOH}, 3-18 \text{ h, reflux}, \text{NaBH}_4 (3.0 \text{ equiv}), 0.5-0.6 \text{ h at rt}; \\
\text{R} \text{CH}_2\text{NH}_2 & \quad (1.2 \text{ equiv}), \text{Xantphos} (0.06 \text{ equiv}), \text{Pd}_2\text{dba}_3 (0.02 \text{ equiv}), \\
\text{NaO} & \quad \text{tBu} (2.5 \text{ equiv}), 1,4\text{-dioxane, MW, } 30 \text{ min, } 100 \text{ °C.}
\end{align*}
\]

B

\[
\begin{align*}
\text{R} \text{NO}_\text{Cl} & \quad \text{EtOH, 6 h, reflux, NaBH}_4 (1.5 \text{ equiv}), \text{EtOH, 6 h, reflux, NaBH}_4 (1.5 \text{ equiv}), \\
\text{R} & \quad \text{R'} = \text{various substituted phenyl and heteroaromatic groups}.
\end{align*}
\]

See Tables 1-3 for details.
RESULTS AND DISCUSSION:

After having confirmed the identity and biological activity of the resynthesized lead compound 1, the NIH and the Holman lab began our systematic SAR explorations as shown in Tables 1-3. Our initial concern surrounding this chemotype was the presence of the catechol-like moiety as this functionality can often be associated with promiscuous activity and/or metal chelation. However, the excellent selectivity of 1 over related lipoxygenase enzymes (5-LOX, 15-LOX-1 and 15-LOX-2) suggested this was not the case. Regardless, we chose to focus on modification of the 2-hydroxy-3-methoxybenzyl moiety for our first round of analog synthesis. As shown in Table 1, we found that removal of the phenolic groups (8), 3-Ome group (9), or the 2-OH group (10) resulted in a complete loss of activity. Protecting the 2-OH moiety as methyl ether (compounds 11-12) or replacing with an amine (compounds 13 and 15) also negated all 12-LOX inhibitory activity. We tried a bioisosteric replacement of the catechol moiety with an indole (17), however this analog was also inactive. These data seemed to indicate the requirement of the 2-OH group, yet this group alone was not enough for activity. Therefore, we decided to hold the 2-OH group constant and try modifications at the other positions. We found the 3-Cl (19, IC50 = 6.2 μM) to have comparable activity, while the 3-fluoro and 3-bromo (20, IC50 = 19
μM and 21, IC₅₀ = 13 μM) were ~4 to ~2.5-fold fold less active, respectively. An interesting and unexpected result was the 2-fold improvement in activity observed for the 4-bromo derivative (22, IC₅₀ = 2.2 μM) and 4-chloro derivative (27), which had comparable activity (6.3 μM). In contrast, the 4-methoxy derivative (28) had reduced activity compared to compound 1 (IC₅₀ = 22 μM). Other substituents at 3-position such as methyl (23), amino (24) and nitro (25) all resulted in a drastic loss of activity IC₅₀ > 40 μM as did all 5-position analogs (29-33). Only one compound with a modification to the 6-position was synthesized, R = OMe (34) and this was also inactive. Thus, we determined the 2-OH to be essential for activity and the 3-position to be most optimal for the methoxy group. However, this preliminary SAR suggested that the 3-OMe could be replaced with a chloro group (19) and a 4-bromo (22) and 4-chloro (27) while maintaining comparable, if not improved activity to compound 1.
Having established a preliminary SAR profile of this region of the molecule, we next turned our attention to modification of the thiazole group as shown in Table 2. Gratifyingly, unlike our efforts on the phenyl moiety, changes to this region of the molecule led to analogs with improved potency. Replacing the thiazole moiety with a 2-benzothiazole (35, ML355) resulted in an 18-fold improvement in 12-LOX activity while retaining selectivity. The benzoxazole (36) and benzimidazole (37) possessed good to excellent activity, and introduction of a methyl group at the 4-position of the benzothiazole ring (39), maintained excellent 12-LOX activity (IC50 = 0.24 μM). Potency against 12-LOX was also improved over 10-fold when the thiazole was...
replaced with a thiophene (38), but the substituted thiazole/isoxazole derivatives (40-43) did not show this increased potency. The phenyl derivative (48), a known thiophene bioisostere, also demonstrated potent activity against 12-LOX (IC$_{50}$ = 0.5 μM). Generally, larger aromatic [1-naphthalene (49) and 2-naphthalene (50)] and heteroaromatic compounds [3-quinoline (46), 8-isoquinoline (47), 2-pyridine (58) and 3-pyridine (59)] were well tolerated and had better potency than the thiazole derivative (1). In an effort to improve solubility by adding solubilizing functionality, we synthesized a few phenyl derivatives substituted with a piperazine moiety at different positions around the aryl ring (53-55). While these changes were tolerated, they had much reduced activity compared to the top actives (e.g. entry 35 and 36).

Given our interest in pursuing multi-parameter optimization, we simultaneously tested many of these analogs for activity against 15-LOX-1 to ensure that selectivity was maintained. These compounds were initially tested against 15-LOX-1 at a single concentration (25 μM) and an IC$_{50}$ was determined only on compounds of particular interest. These studies showed that replacement of the thiazole with a benzothiazole, and its derivatives maintained favorable selectivity with 15/12-LOX ratios of 29-fold (35), 18- (39), 19- (62) and 20- (68). The 15-LOX-1/12-LOX selectivity ratio improved to over 100 with conversion of the benzothiazole to a benzoazole (36), benzimidazole (37) and $m$-iPr substituted phenyl (67). Interestingly, the phenyl substitution (48) only had a selectivity ratio of 15. A wide-range of selectivity was observed despite the compounds bearing comparable structures, ranging from almost complete inhibition of 15-LOX-1 (e.g. 38 and 63) to minimal inhibition (e.g. 55, 58,
and 66). The dramatic effect on selectivity between 12-LOX and 15-LOX-1 in this portion of the molecule supports the differences between the active sites of the two LOX isozymes, as seen previously by mutational analysis.\textsuperscript{xxiv}

Table 2. 12-LOX Inhibition of analogs 35-68\textsuperscript{a}

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>12-LOX IC\textsubscript{50} (\textmu M)</th>
<th>15-LOX IC\textsubscript{50} (\textmu M)</th>
<th>% inh\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>35 (ML365)</td>
<td>2-benzothiazole</td>
<td>0.34</td>
<td>9.7</td>
<td>—</td>
</tr>
<tr>
<td>36</td>
<td>2-benzoxazole</td>
<td>0.79</td>
<td>&gt;100</td>
<td>—</td>
</tr>
<tr>
<td>37</td>
<td>2-benzimidazole</td>
<td>0.57</td>
<td>&gt;100</td>
<td>—</td>
</tr>
<tr>
<td>38</td>
<td>2-thiophene</td>
<td>0.35</td>
<td>—</td>
<td>100</td>
</tr>
<tr>
<td>39</td>
<td>4-Me-2-benzothiazole</td>
<td>0.24</td>
<td>4.2</td>
<td>—</td>
</tr>
<tr>
<td>40</td>
<td>4-Me-2-thiazole</td>
<td>2.8</td>
<td>—</td>
<td>14</td>
</tr>
<tr>
<td>41</td>
<td>5-Me-2-thiazole</td>
<td>3</td>
<td>—</td>
<td>75</td>
</tr>
<tr>
<td>42</td>
<td>5-Ph-2-thiazole</td>
<td>91%\textsuperscript{b}</td>
<td>—</td>
<td>77</td>
</tr>
<tr>
<td>43</td>
<td>4,5-Me-2-thiazole</td>
<td>1.4</td>
<td>—</td>
<td>37</td>
</tr>
<tr>
<td>44</td>
<td>5-Me-3-isoxazole</td>
<td>11</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>45</td>
<td>3-OMe-P</td>
<td>85%\textsuperscript{b}</td>
<td>—</td>
<td>73</td>
</tr>
<tr>
<td>46</td>
<td>3-quinoline</td>
<td>0.48</td>
<td>—</td>
<td>77</td>
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<tr>
<td>47</td>
<td>8-isoquinoline</td>
<td>0.70</td>
<td>—</td>
<td>70</td>
</tr>
<tr>
<td>48</td>
<td>Ph</td>
<td>0.50</td>
<td>7.6</td>
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<tr>
<td>49</td>
<td>1-naphthalene</td>
<td>0.51</td>
<td>—</td>
<td>73</td>
</tr>
<tr>
<td>50</td>
<td>2-naphthalene</td>
<td>0.33</td>
<td>—</td>
<td>54</td>
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<td>51</td>
<td>1,4-bi-Ph</td>
<td>1.3</td>
<td>—</td>
<td>60</td>
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<tr>
<td>52</td>
<td>1,3-bi-Ph</td>
<td>82%\textsuperscript{b}</td>
<td>—</td>
<td>70</td>
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<tr>
<td>53</td>
<td>3-piperazine-Ph</td>
<td>3.5</td>
<td>—</td>
<td>31</td>
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<tr>
<td>54</td>
<td>4-piperazine-Ph</td>
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<td>4-piperidine-Ph</td>
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<td>56</td>
<td>4-piperazine-3-pyr</td>
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<tr>
<td>57</td>
<td>4-methyl-3-pyr</td>
<td>5</td>
<td>—</td>
<td>18</td>
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<tr>
<td>58</td>
<td>2-pyr</td>
<td>5</td>
<td>—</td>
<td>3</td>
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<td>59</td>
<td>3-pyr</td>
<td>7</td>
<td>—</td>
<td>—</td>
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<tr>
<td>60</td>
<td>2-pyrimidine</td>
<td>12</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>61</td>
<td>3-Bu-Ph</td>
<td>0.39</td>
<td>—</td>
<td>66</td>
</tr>
<tr>
<td>62</td>
<td>6-methoxy-2-benzothiazole</td>
<td>0.26</td>
<td>5.1</td>
<td>—</td>
</tr>
<tr>
<td>63</td>
<td>4-Phe-2-thiazole</td>
<td>0.18</td>
<td>—</td>
<td>87</td>
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<tr>
<td>64</td>
<td>3-morpholine-Ph</td>
<td>3.8</td>
<td>—</td>
<td>50</td>
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<tr>
<td>65</td>
<td>4N-boc-piperidine-3-Ph</td>
<td>0.76</td>
<td>—</td>
<td>34</td>
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<tr>
<td>66</td>
<td>3-piperidine-Ph</td>
<td>1.1</td>
<td>—</td>
<td>4.7</td>
</tr>
<tr>
<td>67</td>
<td>3-iPr-Ph</td>
<td>0.16</td>
<td>&gt;100</td>
<td>—</td>
</tr>
<tr>
<td>68</td>
<td>6-F-2-benzothiazole</td>
<td>0.22</td>
<td>4.5</td>
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IC50 values represent the half maximal (50%) inhibitory concentration as determined in the UV-Vis cuvette-based assay in triplicate. The error for these values are below 20%. \(^b\) represents percent inhibition at 25 \(\mu\text{M}\).

As noted above (Table 1), replacement of the “right-hand” portion of the molecule with a 3-chloro-2-phenol (19), 4-bromo-2-phenol (22) or 2-chloro-2-phenol (27), resulted in comparable potency to 1 with IC\(_{50}\) values of 6.2, 2.2 and 6.3 \(\mu\text{M}\) respectively. Therefore, we sought to explore a matrix library utilizing these groups with some of the best sulfonamide derivatives discovered as part of the initial SAR efforts (see Table 3, compounds 69-83). Unfortunately, none of the compounds had improved potency. Generally, the 2-benzothiazole moiety gave the best activity with both the 3-chloro-2-phenol (71; IC\(_{50}\) = 1.3 \(\mu\text{M}\)) and 4-bromo-2-phenol (79; IC\(_{50}\) = 1.7 \(\mu\text{M}\)), although the 1-naphthalene derivative 78 also had comparable potency (IC\(_{50}\) = 1.3 \(\mu\text{M}\)).
The results of our SAR investigations, which provided compounds with low nM potency against 12-LOX, prompted me to examine the selectivity of a few of our top analogs (35, 36, and 37) against other human LOX isozymes (5-LOX and 15-LOX-2). In addition, we profiled the compounds against cyclooxygenase-1/2 (COX-1/2). As shown in Table 4, we observed no significant inhibition against any of these related enzymes, with exception to 35 which has modest potency (29-fold less active).
towards 15-LOX-1. These results are encouraging because few compounds reported in the literature have achieved both nM potency towards 12-LOX and selectivity against other isozymes.

Table 4. Selectivity and Redox Activity of Representative Analogs.

<table>
<thead>
<tr>
<th>Analog</th>
<th>12-LOX&lt;sup&gt;b&lt;/sup&gt;</th>
<th>15-LOX-1&lt;sup&gt;b&lt;/sup&gt;</th>
<th>15-LOX-2&lt;sup&gt;b&lt;/sup&gt;</th>
<th>5-LOX&lt;sup&gt;b&lt;/sup&gt;</th>
<th>COX-1/2&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Redox Activity&lt;sup&gt;d&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>35</td>
<td>0.34</td>
<td>9.8</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>NI</td>
<td>NI</td>
</tr>
<tr>
<td>36</td>
<td>0.79</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>NI</td>
<td>NT</td>
</tr>
<tr>
<td>37</td>
<td>0.57</td>
<td>&gt;100</td>
<td>&gt;100</td>
<td>&gt;35</td>
<td>NI</td>
<td>NT</td>
</tr>
</tbody>
</table>

<sup>a</sup> Selectivity profiling of 35 and other top compounds. <sup>b</sup>IC<sub>50</sub> values are reported in μM. The error for these values are below 20%. <sup>c</sup> Compounds were tested at 15 μM and none of the compound exhibited inhibition above 10%. <sup>d</sup>UV-Vis pseudoperoxidase activity assay was performed on 35 and no degradation of the hydroperoxide product was observed at 234 nm, indicating a non-reductive inhibitory mechanism; NI = no inhibition and NT = not tested.

LOX inhibitors are known to exhibit a variety of inhibitory mechanisms; therefore the UV pseudoperoxidase assay is often used to investigate if the inhibition is reductive in nature.

I performed the assay on 35 and 36 with 12-LOX and no degradation of the hydroperoxide product was observed at 234 nm, indicating a non-reductive inhibitory mechanism (Table 4). To investigate the inhibition mechanism further, I performed steady-state kinetics using both 35 and 36 by monitoring the formation of 12-HPETE as a function of substrate and inhibitor concentration in the presence of 0.01% Triton X-100. Replots of K<sub>m</sub>/V<sub>max</sub> and 1/V<sub>max</sub> versus inhibitor concentration yielded linear trends for both 35 and 36. The K<sub>i</sub> for 35 equaled 0.35 +/- 0.08 mM and 0.53 +/- 0.2 mM for 36, from the K<sub>m</sub>/V<sub>max</sub> graphs.
Figure 2. Steady-state kinetics data for the determination of $K_i$ for 12-LOX with 35. $K_M/V_{max}$ (x-intercept, $K_M/V_{max}$ units are mM/mmol/min/mg) versus [Inhibitor] ($\mu$M) is the secondary replot of the inhibition data, which yielded a $K_i$ of 0.35 +/- 0.08 mM.

Figure 3. Steady-state kinetics data for the determination of $K_i$ for 12-LOX with 36. $K_M/V_{max}$ (x-intercept, $K_M/V_{max}$ units are mM/mmol/min/mg) versus [Inhibitor] ($\mu$M) is the secondary replot of the inhibition data, which yielded a $K_i$ of 0.53 +/- 0.2 mM.
The $K_i$ equaled 0.72 +/- 0.1 mM for 35 and 0.62 +/- 0.1 mM for 36, from the $1/V_{max}$ graphs.

**Figure 4.** Steady-state kinetics data for the determination of $K_i$ for 12-LOX with 35. $1/V_{max}$ (y-intercept, $1/V_{max}$ units are 1/mmol/min/mg) versus [Inhibitor] (µM) is the secondary replot of the inhibition data, which yielded a $K_i$ of 0.72 +/- 0.1 mM.
Figure 5. Steady-state kinetics data for the determination of $K_i$ for 12-LOX with 36. $1/V_{\text{max}}$ (y-intercept, $1/V_{\text{max}}$ units are 1/mmol/min/mg) versus [Inhibitor] (µM) is the secondary replot of the inhibition data, which yielded a $K_i$ of 0.63 +/- 0.1 mM.

The data for both 35 and 36 correlate with their IC$_{50}$ values (Table 2) and indicate that both molecules are mixed inhibitors, which is a common property of both 12-LOX$^{xxi}$ and 15-LOX-1$^{xxvi}$ inhibitors in general.

Upon completion of the SAR campaign, we then sought to explore the activity of 35 in relevant cell-based systems. As noted previously, 12-LOX has been linked to platelet activation, which plays a central role in the regulation of primary hemostasis and arterial thrombosis. Consequently, failure to attenuate platelet activation results in excessive clot formation leading to adverse cardiovascular events such as myocardial infarction and stroke. Previous studies have shown that 12-LOX in human platelets is highly activated following stimulation of the protease-activated receptor-4 (PAR4) by
the PAR4-activating peptide (PAR4-AP).xxvii Moreover, the bioactive metabolite of 12-LOX (12-HETE), resulting from the stereospecific oxidation of AA and reduction by peroxidases, demonstrates pro-thrombotic effects in human platelets.xxviii Therefore, treatment of PAR4-AP-induced human platelets with a small molecule 12-LOX inhibitor should attenuate the platelet aggregation in a dose-dependent manner. The results from the Holinstat Laboratory in Figure 2B show that 35 does in fact significantly reduce PAR4-AP induced platelet aggregation. Further, 12-LOX has been shown to play a role in calcium mobilization in human platelets and inhibition of 12-LOX should lead to a reduced concentration of free calcium in the platelet.xxix To study this, we stimulated human platelets with 200 μM PAR4-AP and measured the free calcium in the platelet, using a C6 Accuri flow cytometer with various concentrations of 35 (Figure 2A). These data demonstrate that at concentrations as low as 250 nM of 35 calcium mobilization is reduced significantly (measured as fold change), with complete inhibition of calcium mobilization occurring at ~5 μM. Comparable results were obtained with another top active (compound 36) and are shown in Figure 6.
Figure 6. PAR4-AP-induced calcium mobilization (A) and platelet aggregation (B) in human platelets. For A: Washed human platelets (1 x 10⁶ platelets/mL) were stimulated with 200 μM PAR4-AP in the absence or presence of increasing concentrations of compound 36. Calcium mobilization was decreased as the concentration of compound 36 was increased. Calcium was measured in real time using a C6 Accuri flow cytometer. The experiments were done in triplicate. For B: Platelet aggregation of human platelets (3 x 10⁸ platelets/mL) was measured in real-time using a Chronolog Lumi-Aggregometer (model 700D) following addition of PAR4-AP.

Figure 7. PAR4-AP-induced calcium mobilization (A) and platelet aggregation (B) in human platelets. For A: Washed human platelets (1 x 10⁶ platelets/mL) were stimulated with 200 μM PAR4-AP in the absence or presence of increasing concentrations of 35. Calcium mobilization was decreased as the concentration of 35 was increased. Calcium was measured in real time using a C6 Accuri flow cytometer. The experiments were done in triplicate. For B: Platelet aggregation of human platelets (3 x 10⁸ platelets/mL) was measured in real-time using a Chronolog Lumi-
A

B

Aggregometer (model 700D) following addition of PAR4-AP. While 10 μM 35 did not inhibit platelet aggregation, 25 μM 35 inhibited 80% platelet aggregation in washed human platelets.

To further validate these compounds, the Nadler laboratory then assessed the ability of 35 to inhibit 12-LOX in cell-based models relevant for diabetic disease. As discussed above, 12-LOX is expressed in pancreatic β cells and its metabolic product, 12-HETE, is implicated in cytokine-induced cell death. Specifically, 12(S)-HETE has been shown to reduce metabolic activity inhibit insulin secretion and ultimately induce cell death in human islets.xxx Towards this end, the Nader laboratory tested 35 in both a mouse derived β-cell line (BTC3) and human primary donor islets to determine its ability to inhibit AA/calcium ionophore induced stimulation of 12-HETE. 35 was able to potently inhibit 12-HETE in BTC3 cells with an approximate IC₅₀ of 1 μM, as measured by ELISA (Figure 3A). Given the difficulty in obtaining primary human islets from donated tissues, the activity in human islets was performed at a single concentration. The data presented in Figure 3B demonstrates significant inhibition of AA/IONO-induced 12-HETE production, at 10 μM of 35.
Figure 8. Inhibition of 12-HETE in mouse beta cells (A) and human islets (B). For A: Mouse beta cells (BTC3) were treated with arachidonic acid and calcium ionophore (AA/IONO) alone or in the presence of 35. Graphed are the levels of 12-HETE expressed as a percentage of that detected in cells stimulated with AA/IONO alone. For B: The graph represents the increase (above control/unstimulated) in 12-HETE for human primary donor islets stimulated with arachidonic acid and calcium ionophore (AA/IONO) alone or in the presence of 10 μM of 35. 12-HETE was measured by ELISA.

While the above data demonstrates activity in disease relevant cell-based models, in order to validate the potential use of 35 in proof of concept animal models, we needed to determine both its in vitro ADME and in vivo PK properties. These data were performed by a contracted laboratory and are summarized in Tables 5 and 6 respectively. Compound 35 demonstrated excellent microsomal stability with both rat (T1/2 >30 minutes) and mouse (T1/2 >300 minutes) and was found to be stable to mouse plasma over a 2 hour period (100% remaining). Moreover, 35 showed no degradation in various aqueous buffers (pH 2-9) and was stable to 5 mM glutathione suggesting excellent stability. One remaining liability is its aqueous solubility, which is <5 μM, however improved solubility is observed in the assay buffer (qualitative analysis). Moreover, we recently found that benzoxazole derivative (36) has much improved solubility in buffer (>10-fold) albeit with slightly weaker potency towards 12-LOX. As a result, we plan to investigate in more detail the ADME/PK properties of compound 36 in future studies.
Table 5. *in vitro* ADME profile for 35. Experiments were conducted at Pharmaron Inc. *a* represents the stability in the presence of NADPH. 35 showed no degradation without NADPH present over a 1 h period.

<table>
<thead>
<tr>
<th>Compound</th>
<th>PBS buffer (pH 7.4) Solubility (µM)</th>
<th>Microsomal Stability</th>
<th>Mouse Plasma Stability remaining at 2 hours</th>
<th>Permeability (10^-6 cm/s)</th>
<th>Efflux ratio</th>
<th>Stability at 48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>35</td>
<td>&lt;5</td>
<td>&gt;30 (rat)</td>
<td>&gt;300&lt;sup&gt;a&lt;/sup&gt; (mouse)</td>
<td>1.5&lt;sup&gt;a&lt;/sup&gt; (A→B)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.8</td>
<td>100%&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Table 6. *In vivo* PK (mouse) at 3 mpk (IV) and 30 mpk PO for 35. All experiments were conducted at Pharmaron Inc. using male CD1 mice (6-8 weeks of age). Data was collected in triplicate at 8 time points over a 24 h period. *a*Formulated as a solution (5% DMSO, 10% Solutol, 20% PEG400, 65% water). *b* represents the time for elimination of 63.2% of the IV dose.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Route&lt;sup&gt;a&lt;/sup&gt;</th>
<th>T&lt;sub&gt;1/2&lt;/sub&gt; (h)</th>
<th>T&lt;sub&gt;max&lt;/sub&gt; (h)</th>
<th>C&lt;sub&gt;max&lt;/sub&gt; (µM)</th>
<th>AUC&lt;sub&gt;0-t&lt;/sub&gt; (µM)</th>
<th>V&lt;sub&gt;a&lt;/sub&gt; (L/kg)</th>
<th>Cl&lt;sub&gt;lin&lt;/sub&gt; (mL/min/kg)</th>
<th>%F</th>
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<td>35</td>
<td>IV</td>
<td>3.4</td>
<td>NA</td>
<td>112</td>
<td>34</td>
<td>0.55</td>
<td>3.4</td>
<td>NA</td>
<td>2.44</td>
</tr>
<tr>
<td></td>
<td>PO</td>
<td>2.9</td>
<td>0.25</td>
<td>38</td>
<td>67</td>
<td>NA</td>
<td>NA</td>
<td>19.8</td>
<td>NA</td>
</tr>
</tbody>
</table>

Compound 35 showed moderate cell permeability in the Caco-2 assay (1.5 x 10<sup>-6</sup> cm/s) and does not appear to be a substrate for Pgp given the efflux ratio of <2. With
this data in hand, we then sought to explore the *in vivo* PK (mouse) properties of the molecule (Table 6). Exploratory formulation studies led to an appropriate vehicle (DMSO:Solutol:PEG400:water; 5/10/20/65 v/v/v/v) in which 35 was administered as a solution via IV (3 mpk) and PO (30 mpk). These studies demonstrate that 35 is orally bioavailable (%F = ~20), with a moderate half-life (T1/2 = 2.9 hours). At 30 mpk dosing, 35 achieves a Cmax of over 135 times the *in vitro* IC50 and remains over IC50 value for over 12 hours. The compound has low clearance (3.4 mL/min/kg) and good overall exposure (AUCinf) of 38 μM. The volume of distribution (Vd) is (0.55 L/kg) which is low but suggests a reasonable distribution between tissue and blood.

Given the favorable microsomal stability (phase I metabolism) yet modest *in vivo* T1/2 we suspected that the phenolic moiety could be glucuronidated (phase II metabolism) leading to higher clearance than anticipated. In fact, incubation with UDPGA co-factor instead of NADPH led to a T1/2 of ~8 minutes (vs. >30 minutes with NADPH). We thought that the sterically hindered environment of the 2-OH would possibly prevent glucuronidation from occurring, yet this data suggests otherwise. Another strategy which has been used to obviate a glucuronidation liability is to introduce electron-withdrawing groups next to the phenolic moiety of interest. As such, we employed this strategy with the synthesis of analog 71 (2-OH, 3-Cl), however, this did not change the rate of glucuronidation. In addition to introducing other electron-withdrawing groups to the ring, another approach is to modify the phenolic hydroxyl using a pro-drug approach. Ideally, the pro-drug would be slowly hydrolyzed to the free phenol, after it has bypassed first-pass metabolism. This approach has been used
successfully in several marketed drugs, which contain phenolic groups. We plan to investigate these various approaches in future studies.

As stated above, previously reported inhibitors of 12-LOX, such as baicalein and nordihydroguaiaretic acid (NDGA), “bromo-phenols” or “pyrazole derivatives” (see Figure 1) all possess several liabilities. These compounds are not only less potent and less selective, but are also not easily amenable to further optimization. Our previously described 12-LOX inhibitor (ML127, Figure 1) does demonstrate potent inhibition (<500 nM) and excellent selectivity but was found to exhibit flat SAR thus providing little opportunity for further modification. In this work we describe a new chemotype that is structurally distinct from all previously reported inhibitors, and possesses a drug-like scaffold. Compound 35, and related top analogs demonstrate potent (<500 nM) inhibition towards 12-LOX and excellent selectivity against related enzymes (15-LOX-1, 5-LOX, 15-LOX-2, COX 1/2). This series is readily amenable to structural modifications and displays clear and tractable SAR. Compound 35 exhibits a favorable in vitro ADME and in vivo PK profile with activity in disease relevant cell-based systems, such as thrombosis (platelet aggregation and calcium mobilization) and diabetes (12-HETE reducing in β-cells). Future studies are aimed at additional biological characterization of other top compounds (e.g. 36, 37 and 67), which possess comparable potency to 35 but with improved selectivity and solubility (e.g. 36). We hope these compounds have the potential to further validate 12-LOX as a potential therapeutic target for a variety of diseases and we make them readily available to the research community.
EXPERIMENTAL SECTION:

All air or moisture sensitive reactions were performed under positive pressure of nitrogen with oven-dried glassware. Chemical reagents and anhydrous solvents were obtained from commercial sources and used as-is. Preparative purification was performed on a Waters semi-preparative HPLC. The column used was a Phenomenex Luna C18 (5 micron, 30 x 75 mm) at a flow rate of 45 mL/min. The mobile phase consisted of acetonitrile and water (each containing 0.1% trifluoroacetic acid). A gradient of 10% to 50% acetonitrile over 8 minutes was used during the purification. Fraction collection was triggered by UV detection (220 nm). Analytical analysis for purity was determined by two different methods denoted as Final QC Methods 1 and 2. Method 1: Analysis was performed on an Agilent 1290 Infinity Series HPLC. UHPLC Long Gradient Equivalent 4% to 100% acetonitrile (0.05% trifluoroacetic acid) in water over 3.5 minutes run time of 4 minutes with a flow rate of 0.8 mL/min. A Phenomenex Kinetex 1.7 micron C18 column (2.1 x 100 mm) was used at a temperature of 50 °C. Method 2: analysis was performed on an Agilent 1260 with a 7 minute gradient of 4% to 100% acetonitrile (containing 0.025% trifluoroacetic acid) in water (containing 0.05% trifluoroacetic acid) over 8 minute run time at a flow rate of 1 mL/min. A Phenomenex Luna C18 column (3 micron, 3 x 75 mm) was used at a temperature of 50 °C. Purity determination was performed using an Agilent Diode Array Detector for both Method 1 and Method 2. Mass determination was performed using an Agilent 6130 mass spectrometer with electrospray ionization in the positive mode. All of the analogs for assay have purity greater than 95% based on both
analytical methods. $^1$H and $^{13}$C NMR spectra were recorded on a Varian 400 (100) MHz spectrometer. High resolution mass spectrometry was recorded on Agilent 6210 Time-of-Flight LC/MS system.

**Methods.**

**General Synthetic Procedures Performed at the NIH.** (Scheme 1, Method A): 4-amino-\(N\)-(thiazol-2-yl)benzene sulfonamide (0.39 mmol) (2), and the required benzaldehyde (0.67 mmol) was added to a microwave vial and ethanol (2 mL) was added. The reaction vessel was sealed and heated to 100 °C for 4-18 h. The reaction mixture was allowed to cool to room temperature and sodium borohydride (0.80 mmol) was added, stirred for 30 min, during which time the reaction turned clear and then cloudy. The resulting solids were filtered, washed with ethanol, and purified using a prep-HPLC (gradient 10-100% acetonitrile w/ 0.1% TFA in water w/ 0.1% TFA) to give the desired product.

(Scheme 1, Method B) A solution of 4-bromo-\(N\)-(thiazol-2-yl)benzene sulfonamide (0.31 mmol) (3) in dioxane (1 mL) was added to a mixture of sodium tert-butoxide (0.78 mmol), 4,5-bis(diphenylphosphino)-9,9-dimethylxanthene (Xantphos) (0.02 mmol) and tris(dibenzyldieneacetone)dipalladium(0) (Pd$_2$dba$_3$) (6.27 μmol) in dioxane (1 mL). The resulting mixture was degassed with argon for 15 min. Then the requisite benzylamine (0.38 mmol) was added, the vessel was sealed and heated to 100 °C for 30 min in Biotage® microwave reactor. The reaction mixture was cooled to room temperature and filtered through celite. Silicycle® silica bound thiol was
added and stirred overnight, again filtered through a pad of celite, concentrated, and purified by prep-HPLC (gradient 10-100% acetonitrile w/ 0.1% TFA in water w/ 0.1% TFA) to give the desired product.

(Scheme 1, Method C) 4-aminobenzenesulfonamide (4) (1.00 g, 5.81 mmol), 2-hydroxy-3-methoxybenzaldehyde (1.00 g, 7.00 mmol) in EtOH (29 mL) was heated to reflux for 6 h until reaction is an orange turbid mixture. The reaction mixture was cooled to room temperature before sodium borohydride (0.33 g, 8.71 mmol) was added and stirred for an additional 30 min. A white solid forms after 30 min and is collected by filtration and washed with copious amounts of ethanol, dried under vacuum and used as is in subsequent reactions. $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 7.60–7.27 (m, 2H), 6.75–6.40 (m, 4H), 6.06 (t, J = 7.63 Hz, 1H), 4.18 (s, 2H), and 3.65 (s, 3H); $^{13}$C NMR (101 MHz, DMSO) $\delta$ 40.37, 55.32, 108.91, 109.42, 109.55, 111.29, 111.40, 121.05, 125.05, 127.49, 129.92, 150.17, 152.36, and 156.94; LC-MS retention time (Method 1): 2.876 min. General procedure: (Step iv) 4-(2-hydroxy-3-methoxybenzylamino)benzenesulfonamide (5) (0.58 mmol), arylbromide (0.70 mmol), K$_2$CO$_3$ (1.45 mmol), N,N’-dimethylethylenediamine (0.29 mmol), and copper(I)iodide (0.03 mmol) in 1,4-dioxane (1.5 mL) were place under N$_2$ and sealed in a 5 mL sealed tube. The reaction was heated to 80 °C for 6 to 8 h and monitored by LC/MS analysis. Upon completion the heterogeneous mixture was cooled to room temperature, filtered, and washed with dioxane. The solution was passed through a thiol cartridge (metal scavenging), diluted with AcOEt and washed with NH$_4$Cl (2X),
water, and brine. The crude material was purified using a prep-HPLC (gradient 10-100% acetonitrile w/ 0.1% TFA in water w/ 0.1% TFA) to give the desired product.

(Scheme 1, Method D: Representative Example) N-(benzo[d]thiazol-2-yl)-4-nitrobenzenesulfonamide (7): (Step V) To a stirred solution of benzo[d]thiazol-2-amine (0.50 g, 3.35 mmol) in pyridine (1.60 mL, 20.08 mmol) was added 4-nitrobenzene sulfonyl chloride (6) (0.82 g, 3.68 mmol) in three equal parts. The reaction mixture was heated for 75 min at 100 °C, cooled to room temperature, after which time a yellow precipitate formed. The reaction mixture was allowed to sit at room temperature for 2 h then the yellow solid was collected by filtration, washed with ethanol, and dried under reduced pressure overnight to give 1.10 g of the desired product 7. 

\[
\begin{align*}
1^H \text{NMR (400 MHz, DMSO-}d_6) & \delta 8.90 (ddt, J = 0.75, 1.63, \text{and } 5.59 \text{ Hz, } 1H), 8.54, 8.41–8.28 (m, 1H), 8.13–7.96 (m, 2H), 7.82 (dq, J = 0.80, \text{and } 7.96 \text{ Hz, } 1H), 7.45–7.34 (m, 1H), 7.31–7.19 (m, 1H); \\
\text{LC-MS retention time (Method 1): } & 3.272 \\
\text{min; HRMS: } m/z (M+H)^+ = \text{(Calculated for C}_{13}\text{H}_{10}\text{N}_3\text{O}_4\text{S}_2, 336.0107) \text{ found } 336.0107.
\end{align*}
\]

4-amino-N-(benzo[d]thiazol-2-yl)benzenesulfonamide: (Step VI) N-(benzo[d]thiazol-2-yl)-4-nitrobenzenesulfonamide (0.20 g, 0.60 mmol), zinc (0.16 g, 2.39 mmol), acetic acid (0.14 mL, 2.39 mmol) was dissolved in MeOH (3 mL), and the mixture heated to 60 °C for 2 h. The heterogeneous mixture was filtered through a pad of celite washed with hot methanol, concentrated and purified using prep-HPLC (gradient 10-100% acetonitrile w/ 0.1% NH₄OH in water w/ 0.1% NH₄OH) to give
the desired product. Alternate nitro reduction: \textit{N}-(benzo[d]thiazol-2-yl)-4-nitrobenzenesulfonamide was dissolved in MeOH/EtOAc/THF (1:1:1) to give a 0.05 M solution passed through the H-Cube Pro® flow reactor using a 10% Pd/C, 70 mm CatCart® at 50 bar and 50 °C for two cycles at 0.9 mL/min. The solution was concentrated to give a pale yellow solid in a quantitative yield. $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 7.49–7.34 (m, 3H), 7.18 (ddd, $J = 0.56$, 1.21, and 8.00 Hz, 1H), 7.03 (ddd, $J = 1.33$, 7.24, and 7.97 Hz, 1H), 6.83 (ddd, $J = 1.20$, 7.25, and 7.68 Hz, 1H), 6.51–6.38 (m, 2H), 5.42 (s, 2H); LC-MS retention time (Method 2): 3.933 min; HRMS: $m/z$ (M+H)$^+$ = (Calculated for C$_{13}$H$_{12}$N$_3$O$_2$S$_2$, 306.0365) found 306.0360.

\textit{N}-(benzo[d]thiazol-2-yl)-4-((2-hydroxy-3-methoxybenzyl)amino)benzenesulfonamide (Step VII, representative example) (35, ML355): 4-amino-N-(benzo[d]thiazol-2-yl)benzenesulfonamide (0.10 g, 0.33 mmol), 2-hydroxy-3-methoxybenzaldehyde (0.075 g, 0.491 mmol) were heated in EtOH (1.5 mL) at reflux for 18 h. The reaction mixture was allowed to cool to room temperature before the addition of NaBH$_4$ (0.04 g, 0.98 mmol), and stirred for an additional 6 h. After this time, the reaction mixture was quenched with methanol and water then stirred for 20 min, the solids were filtered through celite, the filtrate collected, and concentrated under reduced pressure to provide a yellow solid. The crude material was purified using a prep-HPLC (gradient 10-100% acetonitrile w/ 0.1% TFA in water w/ 0.1% TFA) to give the desired product 35. $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 12.86 (s, 1H), 8.73 (d, $J = 0.5$ Hz, 1H), 7.75 (ddd, $J = 0.6$, 1.2, and 7.9 Hz, 1H), 7.54–7.46 (m, 2H), 7.40–7.31 (m, 1H), 7.28–7.16 (m, 2H), 6.93–6.79 (m, 2H), 6.78–
6.55 (m, 4H), 4.23 (d, J = 5.8 Hz, 2H) and 3.78 (s, 3H); $^{13}$C NMR (DMSO-$d_6$) δ ppm 152.4, 147.7, 144.3, 128.2, 125.7, 122.9, 120.4, 119.0, 111.4, 110.9, 56.2 and 40.6; LC-MS retention time (Method 1): 2.260 min; HRMS: $m/z$ (M+H)$^+$ = (Calculated for C$_{21}$H$_{19}$N$_3$O$_4$S$_2$, 441.0817) found 441.0819.

**Biological Reagents:** All commercial fatty acids (Sigma-Aldrich Chemical Company) were re-purified using a Higgins HAlsil Semi-Preparative (5 μm, 250 x 10mm) C-18 column. Solution A was 99.9% MeOH and 0.1% acetic acid; solution B was 99.9% H$_2$O and 0.1% acetic acid. An isocratic elution of 85% A:15% B was used to purify all fatty acids, which were stored at –80 ºC for a maximum of 6 months.

**Human Platelets:** Human platelets were obtained from healthy volunteers within the Thomas Jefferson University community and the Philadelphia area. These studies were approved by the Thomas Jefferson University Institutional Review Board, and informed consent was obtained from all donors before blood draw. Blood was centrifuged at 200 g for 13 min at room temperature. Platelet-rich plasma was transferred into a conical tube containing a 10% acid citrate dextrose solution (39 mM citric acid, 75 mM sodium citrate, and 135 mM glucose, pH 7.4) and centrifuged at 2000 g for 15 min at room temperature. Platelets were resuspended in Tyrode’s buffer (12 mM NaHCO$_3$, 127 mM NaCl, 5 mM KCl, 0.5 mM NaH$_2$PO$_4$, 1 mM MgCl$_2$, 5 mM glucose, and 10 mM HEPES), and the final platelet concentration was adjusted to 3 X 10$^8$ platelets/mL after counting with a ZI Coulter particle counter (Beckman Coulter, Fullerton, CA). Reported results are the data obtained using platelets from at
least three different subjects. Agonists and inhibitors were used at concentrations indicated in the figures and figure legends.


**Performed at UCSC:** Human platelet 12-lipoxygenase (12-LOX), human reticulocyte 15-lipoxygenase-1 (15-LOX-1), and human epithelial 15-lipoxygenase-2 (15-LOX-2), were expressed as N-terminally, His₆-tagged proteins and purified to greater than 90% purity, as evaluated by SDS-PAGE analysis.xxxiii Human 5-lipoxygenase was expressed as a non-tagged protein and used as a crude ammonium sulfate protein fraction, as published previously.xxxiv Iron content of 12-LOX was determined with a Finnigan inductively coupled plasma mass spectrometer (ICP-MS), using cobalt-EDTA as an internal standard. Iron concentrations were compared to standardized iron solutions and used to normalize enzyme concentrations.

**High-throughput Screen Materials Performed at NIH:** Dimethyl sulfoxide (DMSO) ACS grade was from Fisher, while ferrous ammonium sulfate, Xylenol Orange (XO), sulfuric acid, and Triton X-100 were obtained from Sigma-Aldrich.

**12-Lipoxygenase qHTS Assay (AID: 1452) Performed at NIH:** All screening operations were performed on a fully integrated robotic system (Kalypsys Inc, San Diego, CA) as described elsewhere.xxxv Three µL of enzyme (approximately 80 nM 12-LOX, final concentration) was dispensed into 1536-well Greiner black clear-bottom assay plates. Compounds and controls (23 nL) were transferred via Kalypsys PinTool equipped with 1536-pin array. The plate was incubated for 15 min at room
temperature, and then a 1 µL aliquot of substrate solution (50 µM arachidonic acid final concentration) was added to start the reaction. The reaction was stopped after 6.5 min by the addition of 4 µL FeXO solution (final concentrations of 200 µM Xylenol Orange (XO) and 300 µM ferrous ammonium sulfate in 50 mM sulfuric acid). After a short spin (1000 rpm, 15 sec), the assay plate was incubated at room temperature for 30 minutes. The absorbances at 405 and 573 nm were recorded using ViewLux high throughput CCD imager (Perkin-Elmer, Waltham, MA) using standard absorbance protocol settings. During dispense, enzyme and substrate bottles were kept submerged into a +4 ºC recirculating chiller bath to minimize degradation. Plates containing DMSO only (instead of compound solutions) were included approximately every 50 plates throughout the screen to monitor any systematic trend in the assay signal associated with reagent dispenser variation or decrease in enzyme specific activity. Data were normalized to controls, and plate-based data corrections were applied to filter out background noise.

**Lipoxygenase UV-Vis Assay Performed at UCSC:** The inhibitor compounds were screened initially using one concentration point at 25 µM on a Perkin-Elmer Lambda 40 UV/Vis spectrophotometer. The percent inhibition was determined by comparing the enzyme rates of the control (DMSO solvent) and the inhibitor sample by following the formation of the conjugated diene product at 234 nm (ε = 25,000 M⁻¹ cm⁻¹). The reactions were initiated by adding either of 30 nM 12-LOX, 40 nM 15-LOX-1, 200 nM 15-LOX-2 or 5-10 µL of 5-LOX crude extract to a cuvette with a 2 mL reaction buffer constantly stirred using a magnetic stir bar at room temperature
(22 ºC). Reaction buffers used for various lipoxygenase were as follows: 25 mM HEPES (pH 7.3), 0.3 mM CaCl₂, 0.1 mM EDTA, 0.2 mM ATP, 0.01% Triton X-100, 10 µM AA for the crude, ammonium sulfate precipitated 5-LOX; and 25 mM HEPES (pH 7.5), 0.01% Triton X-100, 10 µM AA for 12-LOX, 15-LOX-1 and 15-LOX-2.

The substrate concentration was quantitatively determined by allowing the enzymatic reaction to go to completion in the presence of 15-LOX-2. For the inhibitors that showed more than 50% inhibition at the one point screens, IC₅₀ values were obtained by determining the % inhibition, relative to solvent vehicle only, at various inhibitor concentrations. The data was then plotted against inhibitor concentration, followed by a hyperbolic saturation curve fit (assuming total enzyme concentration [E] << Kᵢ-app, so IC₅₀ ~ Kᵢ-app). It should be noted that all of the potent inhibitors displayed greater than 80% maximal inhibition, unless stated in the tables. Inhibitors were stored at –20 ºC in DMSO.

**Steady State Inhibition Kinetics Performed at UCSC:** The steady-state kinetics experiments were performed with 35 to determine the mode of inhibition. The inhibitor concentrations of 0, 0.2, 0.5 and 1 µM were used. Reactions were initiated by adding substrate (range 1 – 5 µM AA) to approximately 30 nM 12-LOX in a constantly stirring 2 mL cuvette containing 25 mM HEPES buffer (pH 7.5), in the presence of 0.01% Triton X-100. Lipoxygenase rates were determined by monitoring the formation of the conjugated product, 12-HPETE, at 234 nm (ε = 25 000 M⁻¹ cm⁻¹) with a Perkin-Elmer Lambda 45 UV/Vis spectrophotometer. It should be noted that 12-LOX displays higher error in the Kₘ values at low substrate concentrations (< 1
μM) due to the limits of the spectrophotometer. The substrate concentration was quantitatively determined by allowing the enzymatic reaction to proceed to completion, using 12-LOX or 15-LOX-2. Kinetic data were obtained by recording initial enzymatic rates, at varied substrate and inhibitor concentrations, and subsequently fitted to the Henri-Michaelis-Menten equation, using KaleidaGraph (Synergy) to determine the microscopic rate constants, $V_{\text{max}}$ (μmol/min/mg) and $V_{\text{max}}/K_M$ (μmol/min/mg/μM). The kinetic rate constants were subsequently replotted with $K_M/V_{\text{max}}$ and $1/V_{\text{max}}$ versus inhibitor concentration, yielding $K_i$ and $K_i^*$, respectively.

**Pseudoperoxidase Assay Performed at UCSC:** The pseudo-peroxidase activity rates were determined with BWb70c as the positive control, 13-(S)-HPODE as the oxidizing product and 12-LOX or 15-LOX-1 on a Perkin-Elmer Lambda 40 UV/Vis spectrophotometer, as described previously.xxv Activity was determined by monitoring the decrease at 234 nm (product degradation) in buffer (50 mM Sodium Phosphate (pH 7.4), 0.3 mM CaCl$_2$, 0.1 mM EDTA, 0.01% Triton X-100, and 20 μM 13-(S)-HPODE). About 60 nM 12-LOX was added to 2 mL buffer containing 20 μM 13-(S)-HPODE, constantly stirred with a rotating stir bar (22 °C). Reaction was initiated by addition of 20 μM inhibitor (1:1 ratio to product). The percent consumption of 13-(S)-HPODE was recorded and loss of product less than 20% was not considered as viable redox activity. Individual controls were conducted consisting of enzyme alone with product and 35 alone with enzyme. These negative controls formed the baseline for the assay, reflecting non-pseudo-peroxidase dependent
hydroperoxide product decomposition. To rule out the auto-inactivation of the enzyme from pseudo-peroxidase cycling, the 12-LOX residual activity was 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, since 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.

**Cyclooxygenase assay Performed at UCSC:** Roughly 2-5 µg of either COX-1 or COX-2 were added to buffer containing 0.1 M Tris-HCl buffer (pH 8.0), 5 mM EDTA, 2 mM phenol and 1 µM hematin at 37 °C. The selected inhibitors were added to the reaction cell, followed by an incubation of 5 minutes with either of the COX enzymes. The reaction was then initiated by adding 100 µM AA in the reaction cell. Data was collected using a Hansatech DW1 oxygen electrode and the consumption of oxygen was recorded. Indomethaslin and the solvent DMSO, were used as positive and negative controls, respectively and the percent inhibition of the enzyme was calculated by comparing the rates from samples and the controls.

**Platelet Aggregation Performed at Jefferson University:** Washed platelets were adjusted to a final concentration of 3 x 10^8 platelets/mL. Where indicated, platelets were pretreated with 35 or compound 36 for 10 min at the indicated concentrations for 1 min. The aggregation response to PAR4-AP was measured using an aggregometer with stirring at 1100 RPM at 37 °C.
**Calcium Mobilization Performed at Jefferson University:** Platelets were recalcified to a final concentration of 1 mM followed by pre-incubation with Fluo-4 AM for 10 min. The platelets were then treated with 35 or compound 36 for 10 min at the indicated concentrations before stimulation with the indicated agonist. Calcium mobilization was measured using the Accuri C6 flow cytometer.

**Mouse Beta cells (12-HETE Inhibition) Assay Performed at East Virginia Medical School:** Cells were gowned to 90% confluency in 24 well plates in DMEM (Cat# 11885092, Life Technologies Grand Island, NY) +10% FBS. Cells were pre-treated with 35 and stimulated as for human islets. After four hours, the media was removed and spun at 1000 RPM for 5 minutes. The cleared supernatant was stored at –80 ºC prior to analysis. For analysis, supernatants were extracted on SepPak c18 SPE column (Cat# WAT054945, Waters Corporation, Milford, MA) and dried under nitrogen gas before reconstitution in 500 μL of 12-HETE ELISA buffer and analysis following manufacturers recommendations (Cat# 901-050, Enzo Life Sciences, Plymouth Meeting, PA).

**Human Islet (12-HETE Inhibition) Assay Performed at East Virginia Medical School:** Human donor islets obtained from integrated islet distribution program (www.iidp.coh.org) were incubated overnight in CMRL media (Cat# 15-110-CV MediaTech, Inc. Manassas, VA) containing 10% Fetal Bovine Serum, 1U penicillin, 1 µg streptomycin (pen/strep). Islets were equilibrated in serum free media, (CMRL containing pen/Strep and 1% fatty acid free human serum albumin (Cat# A1887
Sigma, St. Louis, MO)), for 1 hour prior to pretreatment with 10 μM of 35 for 30 min. For 12-HETE induction, islets were treated with 100 μM arachidonic acid (Cat# BML-FA003-0100, Enzo Life Sciences Plymouth Meeting, PA), and 5 μM A23187 (Cat# C7522, Sigma, St. Louis, MO), for 4 hours at 37 ºC. Islets were harvested, centrifuged at 1000 RPM for 5 minutes with cleared supernatant and islet pellet being stored at -80 ºC. For extraction of the supernatants, samples were acidified to pH 3 with 1N HCl for 30 minutes and spun at 1000 RPM for five minutes. Samples were added to a prepared column (prewashed with 3 mL EtOH, followed by 3 mL of H2O) and washed with 3 mL H2O, followed by 3 mL 15% EtOH, and 3 mL Hexane. The samples were eluted with 3 mL of ethyl acetate and dried under nitrogen gas before, reconstitution in 500 mL of 12-HETE ELISA sample buffer (Enzo Life Sciences, Plymouth Meeting, PA). Cell pellets were extracted using CHCl3 / MeOH and samples dried under nitrogen gas before reconstitution in 250 μL of ELISA sample buffer. 12-HETE levels in samples were determined using a 12-HETE ELISA kit (Cat# 901-050, Enzo Life Sciences, Plymouth Meeting, PA).

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