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Inhibition of Endocannabinoid Metabolism by the Metabolites of Ibuprofen and Flurbiprofen

Jessica Karlsson*, Christopher J. Fowler

Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) mediate their anti-inflammatory, analgesic and antipyretic action by inhibiting production of prostaglandin intermediates by inhibition of cyclooxygenase isoenzymes 1 and/or 2 (COX-1, COX-2) [1]. NSAIDs are widely used although their ability to produce serious unwanted cardiovascular and gastrointestinal effects means that there is a need for novel compounds with an improved safety profile.

The endocannabinoid system has been implicated in the mechanism of NSAIDs (reviewed in [2]). The endocannabinoids anandamide (arachidonylethanolamide, AEA) and 2-arachidonoylglycerol (2-AG) are substrates for COX-2 but not COX-1. Cyclooxygenation producing prostaglandin -ethanolamides and -glycerol esters [3–5]. Ibuprofen is a potent noncompetitive COX-2 inhibitor of 2-AG cyclooxygenation (IC50: 210 nM) but a weak, competitive inhibitor of arachidonic acid (AA) oxygenation (IC50: 180 μM) and this property is shared by other profens such as flurbiprofen [6–8]. In our hands, flurbiprofen inhibits 2-AG oxygenation with an IC50 value of 1.3 μM. The corresponding values for inhibition of AA oxygenation by COX-1 and -2, respectively, are 3.6 and 103 μM [8]. Thus, the traditional description of flurbiprofen as a COX inhibitor with some selectivity for COX-1 over COX-2 is only true when AA is used as substrate. Mechanistically, this is because COX-2 is a dimer with half-sites reactivity. Binding of the NSAID to the allosteric site of the homodimer prevents endocannabinoid oxygenation, whereas inhibition of AA oxygenation requires NSAID binding to the catalytic site [6,7]. Differences in affinities for the two sites account for the substrate-selectivity. More recently, an indomethacin analogue with pronounced selectivity for inhibition of COX-2 catalysed endocannabinoid oxygenation vs. AA oxygenation has been described. This compound increases endocannabinoid levels in the brain in vivo and produces potentially beneficial effects in models of anxiety [9], thus demonstrating that the ability of COX-2 to metabolise endocannabinoids is a physiologically relevant pathway, at least in the brain.

In addition to the effects upon COX isoenzymes, the profens inhibit the hydrolysis of AEA by the enzyme fatty acid amide hydrolase (FAAH) [8,10–14]. Although the potencies are relatively low (e.g. 270 μM for ibuprofen [10]), they are increased 3–4-fold at low pH [15,16]. A lowered extracellular pH (such as is seen in inflamed tissue [17]) is sufficient for this to be seen [18]. Given the ability of acidic NSAIDs to accumulate in inflamed tissue [19], it is
possibility that local inhibition of FAAH can contribute to the effects of the profens in such tissues. Certainly, locally administered ibuprofen synergizes with AEA in rat models of pain and inflammation [20]. The synergistic effect is antagonized by the cannabinoid CB1 inverse agonist AM251 suggesting inhibited AEA metabolism as the mechanism [20]. Indomethacin is acidic and accumulates in inflamed tissue [19], and reduces carrageenan-induced oedema in a manner blocked by the CB2 receptor inverse agonist SR144528 [21], a finding consistent with a mobilization of the endocannabinoid system under these conditions.

Research into the effects of NSAIDs on the endocannabinoid system and the balance between arachidonic acid and endocannabinoid metabolism has focused entirely on the parent compounds and/or their enantiomers. The effects of their major metabolites have not been considered. This is of importance, given that many drugs have active metabolites that contribute to the pharmacological effects of the drugs in vivo. Ibuprofen is metabolized by phase I oxygenation to the major metabolites carboxyibuprofen, and 2’-hydroxyibuprofen [22] and minor metabolites 3’-hydroxyibuprofen and 1’-hydroxyibuprofen [23] (structures, see Fig. 1). 4’-hydroxyflurbiprofen is one of the major metabolites of flurbiprofen in rat and human [24] (structures, see Fig. 1). In theory, an active metabolite could show a useful profile, such as a potent inhibition of FAAH and/or blockade of 2-AG oxygenation, but not arachidonic acid oxygenation, by COX-2. In consequence, in the present study, we have compared the potencies of these metabolites with the parent compounds upon the substrate-selective inhibition of COX and the pH-dependent inhibition of FAAH.

**Methods**

**Compounds**

Ibuprofen ((±)-2-(4-Isobutylphenyl)propanoic acid), 1’-hydroxyibuprofen (2-[4-(1-hydroxy-2-methylpropyl)phenyl]propionic acid), 2’-hydroxyibuprofen (2-[4-(2-hydroxy-2-methylpropyl)phenyl]propionic acid), 3’-hydroxyibuprofen (2-[4-(3-hydroxy-2-methylpropyl)phenyl]propionic acid), carboxyibuprofen (2-[4-(2-carboxypropyl)phenyl]propionic acid), flurbiprofen ((±)-2-fluoro-α-methyl[1,1’-biphenyl]-4-acetic acid) and fatty acid-free bovine serum albumin were obtained from Sigma Aldrich (St Louis, MO, USA.). 4’-Hydroxyflurbiprofen (2-(4-hydroxy-2-fluoro-4-biphenyl)propionic acid) was obtained from Santa Cruz Biotechnology (Heidelberg, Germany). The profens were dissolved in DMSO.

![Figure 1. Structures of ibuprofen, flurbiprofen and the metabolites investigated in the present study.](https://example.com/figure1.png)
Figure 2. Inhibition of the activities of ovine COX-1 (towards arachidonic acid) and human recombinant COX-2 (towards either arachidonic acid or 2-AG) by ibuprofen and its 2'- and 3'-hydroxy metabolites. The substrates used (10 μM) are shown in the figure, as are the concentrations (in μM) of the test compounds. "F30" refers to 30 μM flurbiprofen, used as a positive control. Values are means ± s.e.m. (unless enclosed by the symbols), n = 3, of the change in oxygen utilisation following addition of enzyme to the oxygen electrode chamber.

doi:10.1371/journal.pone.0103589.g002

A. Flurbiprofen

COX-1

COX-2

Arachidonic acid

2-AG

B. 4’-OH-Flurbiprofen

COX-1

COX-2

Arachidonic acid

2-AG

Figure 3. Inhibition of the activities of ovine COX-1 (towards arachidonic acid) and human recombinant COX-2 (towards either arachidonic acid or 2-AG) by flurbiprofen and its 4'-hydroxy metabolite. The substrates used (10 μM) are shown in the figure, as are the concentrations (in μM) of the test compounds. Values are means ± s.e.m. (unless enclosed by the symbols), n = 3, of the change in oxygen utilisation following addition of enzyme to the oxygen electrode chamber.

doi:10.1371/journal.pone.0103589.g003
Preparation of rat brain homogenates

Brains (minus cerebella) from adult Wistar or Sprague-Dawley stored at −80°C were thawed, weighed and homogenized in cold buffer (20 mM HEPES, 1 mM MgCl₂, pH 7.0). Homogenates were centrifuged at 35,000 g at 4°C for 20 min and the pellet was resuspended in homogenization buffer (repeated twice) before being incubated at 37°C for 15 min to degrade any endogenous substrate that might interfere with the FAAH assay. Following centrifugation, the pellets were resuspended in cold buffer (50 mM Tris-HCl, 1 mM EDTA, 3 mM MgCl₂, pH 7.4). The protein concentration of each homogenates was determined according to the Bradford assay [25] after which the samples were frozen in liquid N₂ until used for FAAH assay. Ethical permission for the animal experiments was obtained from the local animal research ethical committee (Umeå, Sweden).

Cyclooxygenase assays

The assay was performed according to Meade et al. [26] with minor modifications [27] using an oxygen electrode chamber with integral stirring (Oxgraph System, Hansatech Instruments, King’s Lynn, U.K.). The electrode was calibrated to ambient temperature and air pressure. Samples were prepared in 0.1 M Tris-HCl buffer (20 mM HEPES, 1 mM MgCl₂, pH 7.0) containing 1 mM EDTA, 10 μM substrate (AA or 2-AG) (final assay volume was 2 ml). After addition of test substance dissolved in DMSO (final assay concentration 5%) at an ambient temperature of 4°C, reactions were initiated by addition of AEA-[3H] (assay concentration 0.5 μM) in 10 mM Tris-HCl, 1 mM EDTA, pH 7.4 containing 10 μg ml⁻¹ fatty acid-free bovine serum and incubation at 37°C which was allowed to proceed for 10 min. The DMSO concentration did not affect the observed enzyme activity under the conditions used. Blank samples contained buffer instead of homogenate and DMSO (5%). The assay volume of each sample was 200 μl. The pH values given in the text were determined by measurement at 37°C of scaled-up mixtures of homogenization and assay buffers. The reaction was terminated by addition of 400 μl activated charcoal (80 μl activated charcoal +320 μl 0.5 M HCl) and placement of samples on ice. Samples were kept in room temperature for 45–60 min before being centrifuged at 2500 rpm for 10 min. Aliquots (200 μl) of the supernatant was analyzed for tritium content by liquid scintillation with quench correction.

Statistical Analysis

FAAH inhibition was calculated using log(inhibitor)/response with variable slope (four parameters) algorithm in the GraphPad Prism computer program (GraphPad Software Inc., San Diego, CA, USA). Data was expressed as % of control and p<0.05 and IC₅₀ values where calculated from curves generated from uninhibited (top) values set at 100% and letting residual activity (bottom) values be fixed to zero or floating. The two curves were compared by Akaike’s informative criteria and the best fit was chosen. Initial COX activities were calculated as the change in oxygen tension between 10 and 30 seconds after addition of enzyme.

Results

Inhibition of COX cyclooxygenation by ibuprofen, flurbiprofen and their metabolites

The effects of ibuprofen, flurbiprofen and their metabolites upon the activity of COX-1 (vs. AA) and COX-2 (vs. both AA and 2-AG) are shown in Figs. 2, 3, S1 and S2, and summarized in Tables 1 and 2. Activity was measured as change in oxygen concentration after initiation of reaction. As expected, 300 μM ibuprofen inhibited 98% of COX-1-catalysed oxygenation of arachidonic acid. The corresponding inhibition was 19, 9, 3 and 4%, respectively, for 1’-hydroxy-, 2’-hydroxy-, 3’- hydroxy- and carboxy- ibuprofen metabolites. Complete inhibition of COX-1 was produced by 30 μM flurbiprofen, while its metabolite 4’-hydroxyflurbiprofen produced a 3, 28 and 94% inhibition at 100, 300 and 1000 μM, respectively.

Flurbiprofen (3 μM) inhibited 33% of the COX-2-catalysed oxygenation of arachidonic acid. The corresponding values for oxygenation of 2-AG at 3 μM was 78%, confirming the substrate selective inhibition of COX-2 by this compound [7,8].

<p>| Table 1. Comparison of the effects of the NSAIDs and their metabolites upon the initial activity of COX-1. |</p>
<table>
<thead>
<tr>
<th>NSAID</th>
<th>COX-1 activity (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100 μM</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>26±5</td>
</tr>
<tr>
<td>1’-OH-Ibuprofen</td>
<td>112±21</td>
</tr>
<tr>
<td>2’-OH-Ibuprofen</td>
<td>102±17</td>
</tr>
<tr>
<td>3’-OH-Ibuprofen</td>
<td>81±16</td>
</tr>
<tr>
<td>Flurbiprofen</td>
<td>−3±7</td>
</tr>
<tr>
<td>4’-OH-Flurbiprofen</td>
<td>97±31</td>
</tr>
</tbody>
</table>

Initial activities were calculated as the change in oxygen tension between 10 and 30 seconds after start of reaction. The values are means ± s.e.m, n = 3. doi:10.1371/journal.pone.0103589.t001
Table 2. Comparison of the effects of the NSAIDs and their metabolites upon the initial activity of COX-2.

<table>
<thead>
<tr>
<th>NSAID (μM)</th>
<th>3</th>
<th>30</th>
<th>100</th>
<th>300</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA</td>
<td>2-AG</td>
<td>AA</td>
<td>2-AG</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>79±12</td>
<td>26±2</td>
<td>91±8</td>
<td>103±22</td>
</tr>
<tr>
<td>1'-OH-Ibuprofen</td>
<td>98±17</td>
<td>125±24</td>
<td>83±12</td>
<td>115±21</td>
</tr>
<tr>
<td>2'-OH-Ibuprofen</td>
<td>93±14</td>
<td>105±19</td>
<td>139±41</td>
<td></td>
</tr>
<tr>
<td>3'-OH-Ibuprofen</td>
<td>132±16</td>
<td>105±19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Carboxyibuprofen</td>
<td>132±16</td>
<td>105±19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flurbiprofen</td>
<td>89±6</td>
<td>30±15</td>
<td>88±16</td>
<td>39±11</td>
</tr>
<tr>
<td>4'-OH-Flurbiprofen</td>
<td>117±15</td>
<td>105±24</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Initial activities were calculated as the change in oxygen tension between 10 and 30 seconds after start of reaction. The values are means ± s.e.m., n = 3.

doi:10.1371/journal.pone.0103589.t002
Figure 4. Inhibition of FAAH activity in rat brain homogenates by ibuprofen and its 1'-OH, 2'-OH, 3'-OH and carboxy metabolites. The AEA assay concentration was 0.5 μM. Values are means ± s.e.m. (unless enclosed by the symbols), n = 3. doi:10.1371/journal.pone.0103589.g004

Figure 5. Inhibition of FAAH activity in rat brain homogenates by A, flurbiprofen and its 4'-hydroxy metabolite. The AEA assay concentration was 0.5 μM. Values are means ± s.e.m. (unless enclosed by the symbols), n = 3. doi:10.1371/journal.pone.0103589.g005
channel are essential for the enzymatic activity of the enzyme [31]. Flurbiprofen binds to the channel of COX-1 with its carboxyl group interacting with one of only two polar residues within the channel [31,32]. The polar residue plays a major role in the peroxidase activity and is essential for inhibition by NSAIDs. Thus mutation of this residue reduces the affinity for AA as a substrate and disrupts inhibition by NSAIDs containing a carboxyl group [32]. 4-Hydroxyflurbiprofen is hydroxylated in position 4 of the first phenyl-group. This may add to the bulkiness of the structure and disturb ability for interaction within the narrow channel of COX-1. COX-1 and COX-2 are 67% identical with very high identity in the catalytic domain [33]. They differ in only a few residues of the active site. However, these residues cause a second pocket in the COX-2 structure very close to the NSAID binding site and may account for inhibitor selectivity [33]. Thus, 4'-hydroxyflurbiprofen can still enter the active site. COX-2 utilizes both AA and endocannabinoids as substrates, and this feature may be linked to flexibility in the active site due to rotamer conformations of leucin in position 531 not seen in the narrow channel of COX-1 [34]. Substrate selectivity for 2-AG when inhibited with 4'-hydroxyflurbiprofen and 3'-hydroxyibuprofen may arise due to half-sites reactivity that induces a conformational change limiting the flexibility in leu-531 and thus limiting the possibility for multiple substrates.

AEA is primarily hydrolysed by FAAH, an enzyme with a pH optimum in the region of 8-9 [35]. However, an N-acylthanolamine acid amidase with a pH optimum of 5 and capable of hydrolyzing AEA has also been described [36]. The selective FAAH inhibitor URB597 lacks activity towards NAAA [37] and is thus a good pharmacological tool to assess whether NAAA contributes to AEA hydrolysis. In rat brain homogenates assayed using the same methodology as here, the selective FAAH inhibitor URB597 completely inhibits AEA hydrolysis at both pH 6 and 8 [38] indicating that FAAH is the major contributor to AEA hydrolysis under the conditions used here. The metabolites of both ibuprofen and flurbiprofen were less potent than the parent compounds towards inhibition of FAAH, but they retained the pH-sensitivity of the parent compounds reported previously [15,16,18]. The results give structural insight in how changes in the profen molecule may affect the biological activity and the possibilities of this should be explored. Carpofen binds FAAH at the entrance of the active cleft with the carboxyl-group pointing outwards from the enzyme [39]. A common feature for many potent FAAH inhibitors within the family of NSAIDs are a carboxyl-group together with a phenyl ring often coupled to a chloride atom and a fused aromatic-system [39]. The phenyl group of flurbiprofen points inward the active cleft. A small change in that structure, such as the 4'-hydroxylation of the phenyl-substituent would affect how the molecule fits the active cleft and thus the ability to bind and inhibit the enzyme. Presumably similar processes can explain the loss of potency of the ibuprofen metabolites towards FAAH.

With respect to the increased potency of the compounds towards FAAH as the pH is reduced, the simplest explanation is that the non-ionised form of the compounds (at the carboxyl group, which is retained by the metabolites) are primarily responsible for the inhibition; this would certainly explain why profens are more potent as inhibitors of FAAH in intact cells when the extracellular pH is reduced [18]. However, the increase in potency as the pH is reduced is less than would have been predicted from the Henderson-Haselbach equation. Our working hypothesis is that the observed sensitivity to inhibition is a composite of the selectivity of the non-ionised form of the profens to inhibit the enzyme coupled with a reduced sensitivity of the enzyme to inhibition per se as the pH is reduced. This is consistent with the finding that the N-(3-methylpyridin-2-yl)amide derivative of ibuprofen, which no longer has a carboxyl group, is in fact a slightly less potent inhibitor of FAAH at pH 6 than at pH 8 [40].

A final question concerns whether the metabolites possess sufficient potency towards FAAH (and possibly COX-2 oxygenation of 2-AG for 4'-hydroxyflurbiprofen) to contribute to the effects of the parent compounds in vivo. Extrapolation from in vitro data to the situation in vivo is difficult, to say the least. In the case of flurbiprofen, a dose of 50 mg produces maximal plasma concentrations of R- and S-enantiomers corresponding roughly to a concentration of racemate of 35 μM [41]. Peak plasma ibuprofen concentrations of in the range 110–150 μM have been reported after two 200 mg single doses of two different ibuprofen preparations [42]. Of course, profens bind avidly to plasma proteins, but the FAAH assays are conducted in the presence of fatty acid-free bovine serum albumin, so the conditions are reasonably close. Carboxyibuprofen is the most abundant metabolite in human plasma 3 h after a single dose of 200 mg ibuprofen [22]. Urinary recovery of carboxyibuprofen and 2'-hydroxyflurbiprofen compared to ibuprofen, is 40, 23 and 11%, respectively, over a period of 24 h after administration of 400 mg

Table 3. pH50 and IC50 values for ibuprofen, flurbiprofen and their metabolites towards the inhibition of 0.5 μM [3H]AEA hydrolysis by rat brain FAAH.

<table>
<thead>
<tr>
<th>Compound</th>
<th>pH 6.0 (μM)</th>
<th>pH 7.3 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ibuprofen</td>
<td>4.15±0.05</td>
<td>3.59±0.05</td>
</tr>
<tr>
<td>1'-OH-Ibuprofen</td>
<td>3.47±0.07</td>
<td>47±0.8% inhibition @ 1000 μM</td>
</tr>
<tr>
<td>2'-OH-Ibuprofen</td>
<td>3.42±0.06</td>
<td>15±7% inhibition @ 1000 μM</td>
</tr>
<tr>
<td>3'-OH-Ibuprofen</td>
<td>3.70±0.03</td>
<td>3.01±0.04</td>
</tr>
<tr>
<td>Carboxyibuprofen</td>
<td>3.38±0.08</td>
<td>31±4% inhibition @ 1000 μM</td>
</tr>
<tr>
<td>Flurbiprofen</td>
<td>4.56±0.05</td>
<td>4.29±0.04</td>
</tr>
<tr>
<td>4'-OH-Flurbiprofen</td>
<td>4.07±0.05</td>
<td>3.50±0.03</td>
</tr>
</tbody>
</table>

Values were determined using the log(inhibitor) vs. response – variable slope (four parameters) algorithm in the Prism 6 computer program, with top and bottom values constrained to 100% and 0%, respectively. In all cases, Akaike’s informative criteria indicated that the curves were better fitted to these constraints than the equivalent algorithm where the bottom value was not constrained.

doi:10.1371/journal.pone.0103589.t003
of ibuprofen [43]. 3'-Hydroxyibuprofen, however, belongs to the minor metabolites and is thus unlikely to reach concentrations sufficient for FAAH/COX inhibition in vivo. The same is presumably true for 4'-hydroxyibuprofen, although this metabolite accounts for 43% of the dose recovered in the urine compared to 23% for the parent flurbiprofen after a dose of 50 mg a day for 10 consecutive days [24]. In conclusion, we report that 4'-hydroxyibuprofen, the major metabolite of flurbiprofen inhibit FAAH and COX-2 oxygenation of 2-AG in a manner similar to the parent compound, albeit with a lower potency. Ibuprofen metabolites fail to inhibit FAAH and COX at relevant concentrations and thus are unlikely to contribute to the therapeutic effect and adverse events related to the drug.

Supporting Information

Figure S1 Inhibition of the activities of ovine COX-1 (towards arachidonic acid) and human recombinant COX-2 (towards either arachidonic acid or 2-AG) by 1'-hydroxyibuprofen and carboxyibuprofen. The substrates used (10 mM) are shown in the figure, as are the concentrations (in mM) of the test compounds. Values are means ± s.e.m. (unless enclosed by the symbols), n = 3, of the change in oxygen utilisation following addition of enzyme to the oxygen electrode chamber. (TIF)

Figure S2 Inhibition of the activities of human recombinant COX-2 (towards either arachidonic acid or 2-AG) by the metabolites of ibuprofen. The substrates used (10 mM) are shown in the figure, as are the concentrations (in mM) of the test compounds. Values are means of two experiments of the change in oxygen utilisation following addition of enzyme to the oxygen electrode chamber. (TIF)

Author Contributions

Conceived and designed the experiments: JK CJF. Performed the experiments: JK. Analyzed the data: JK CJF. Contributed to the writing of the manuscript: JK CJF.

References