Endocannabinoid metabolism: The impact of inflammatory factors and pharmacological inhibitors

Jessica Karlsson
To my family
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Original papers

The present thesis is based on the following papers and manuscript, in the text referred to by their Roman numerals.


Abstract

The endocannabinoid (eCB) system is an endogenous signaling system consisting of ligands (referred to as endocannabinoids, eCBs), receptors and metabolic enzymes. The eCB system is involved in homeostatic control of a variety of biological functions such as neuronal signaling, mood, appetite and pathological conditions such as pain, inflammation and tumour progression. The main eCBs N-arachidonoylethanolamine (AEA, anandamide) and 2-arachidonoylglycerol (2-AG) are synthesised upon stimuli when and where their action is demanded. The signaling is brief and the eCBs are quickly degraded. The enzyme primarily responsible for eCB degradation is fatty acid amide hydrolase (FAAH) for AEA and monoacylglycerol lipase (MAGL) for 2-AG. In addition, both substances are substrates for cyclooxygenase-2 (COX-2). COX-2 is upregulated in inflammation, pain and in several tumours including prostate cancers, but it is not known whether COX-2 contribute significantly to eCB metabolism under these conditions.

Increasing endogenous levels of eCBs by inhibiting their degradation is exploited as a future therapy for pain conditions. One suggested therapeutic strategy is dual inhibition of enzymes FAAH and COX-2 to raise AEA levels. Paper I and II of this thesis investigates FAAH and COX inhibitory effects of: the major metabolites and enantiomers of derivatives (flu-AM1 and ibu-AM5) of the current clinically used NSAIDs ibuprofen and flurbiprofen. The metabolites 3´hydroxyibuprofen and 4´hydroxyflurbiprofen retained the FAAH and COX- inhibitory effects seen by the parent compounds although at lower potencies. Both enantiomers of flu-AM1 were equally potent as FAAH inhibitors and displayed a useful substrate selective COX-2 inhibition profile, favoring eCBs as substrates rather than arachidonic acid.

Paper III explores the impact of COX-2 and the effect of (R)-flu-AM1 upon AEA levels and degradation in mouse leukemic macrophage RAW264.7 cells. Despite the high inhibitory potency in enzyme assays, neither (R)-flu-AM1 nor the combination of a FAAH inhibitor with flurbiprofen increased AEA levels in the intact cells to any great extent. This suggests that the eCB turnover in these cells is rather slow. Further, in paper IV, induction of COX-2 did not unmask an ability of this enzyme to “gate” the uptake of AEA analogous to that seen with FAAH.
Paper IV and V focus upon the role of the eCB system in prostate cancer. The eCB system is altered in cancer and is linked to the progression and prognosis of prostate cancer. How and whereby this change occurs is unknown. This thesis explores the impact of the inflammatory factors TNFα, IL-6 and lactic acid induced low pH upon the mRNA levels of eCB related enzymes and the functional impact upon AEA degradation in human DU145 and rat AT-1 prostate cancer cells. TNFα treatment of DU145 and IL-6 and lactic acid induced low pH exposure of AT-1 changed the mRNA levels of 2-AG related enzymes leaving AEA rather unaffected other than for a substantial induction of COX-2 mRNA in DU145 cells. Thus, AEA homeostasis was not shifted in prostate cancer cell lines exposed to inflammatory factors. The results suggest that COX-2 does not gate the uptake of AEA and is a minor contributor to AEA degradation in intact cells.
Populärvetenskaplig sammanfattning

Endokannabinoid (eCB) systemet är ett signalsystem i och mellan celler, involverat i en rad biologiska processer i kroppen. Bland annat verkar eCB systemet smärt-lindrande, aptit-ökande och anti-inflammatoriskt, men det är även involverat i tumörutveckling. De mest kända eCB signalbärarna är anandamid (AEA) och 2-arakidonoylglycerol (2-AG), dessa verkar genom att binda till mottagarmolekyler (framförallt CB₁ och CB₂). Bindning till mottagar-molekylerna begränsas tidsmässigt genom snabb nedbrytning av signalbärarna. Detta innebär att förhindrad nedbrytning skulle kunna leda till förlängd eCB signalering, vilken skulle kunna utnyttjas terapeutiskt.

Studierna vilka presenteras i denna avhandling fokuserar på AEA. AEA bryts framförallt ned av enzymet fettsyra-amidohydrolas (FAAH) men även av cyklooxygenas-2 (COX-2) samt N-acyletanolamin-amidohydrolas (NAAA) i mindre utsträckning. Hur stor betydelse COX-2 har för nedbrytningen av AEA i intakta celler är relativt outforskat. Mängden COX-2 ökar vid inflammation och i flera tumörtyper vilket stödjer en betydande roll för kontroll av nivåerna av AEA i dessa vävnader. I tumörer ses en störd balans i nivåerna av receptorer samt enzymer ansvariga för produktion och nedbrytning av eCB, flertalet av dessa förändringar korrelerar med sjukdomsprognosen. Varför eller hur denna obalans uppstår är dock okänd. Hypotesen som utforskas i denna avhandling lyder att förändringarna induceras av ämnen i tumörcellens närmiljö, exempelvis (ex.) inflammatoriska faktorer. Studierna i denna avhandling undersöker effekten av inflammatoriska faktorer på uttrycket av eCB relaterade enzymer och receptorer i prostata cancer.

Genom att minska aktiviteten i de enzymer som bryter ned AEA ämnar framtidstå lækemedel höja nivåerna av AEA och därmed lindra ex. smärta. För att ytterligare öka möjligheten till effekt önskar man minska aktiviteten i två enzymer istället för ett, genom så kallade "kombinations hämmare". I denna avhandling studeras substanser i avseende på effekt på FAAH och COX-2 i syfte att hitta en "kombinations hämmare" (studie I-III), samt betydelsen av COX-2 på nedbrytningen av AEA i cellmodeller av inflammation och prostatecancer (studie III-IV).
Resultaten visar att de främsta nedbrytningsprodukterna av ibuprofen och flurbiprofen inom gruppen (kända och terapeutiskt välanvända) NSAIDs hämmade FAAH och COX-2 med samma profil som sina modersubstanser men med lägre styrka. Vidare var den nyligen tillverkade substansen \((R)-\text{flu-AM1}\) en stark FAAH/COX-2 hämmare i enzym-aktivitets experiment. Men \((R)-\text{flu-AM1}\) påverkade inte AEA nivåerna i laborativa cellmodeller. Andra försök visade att även förmågan av COX-2 att påverka hastigheten varvid AEA tas upp i cellerna var minimal, till skillnad från FAAH. Sammantaget tyder detta på att COX-2 spelar en mindre roll för nivåerna av AEA i dessa celler.

Inflammatoriska faktorer påverkade uttrycket av några 2-AG relaterade enzymer i prostatacancer celler medan AEA relaterade enzymer och nedbrytningen av AEA förblev opåverkad. Vidare studier behövs för att undersöka hur interaktionen mellan tumörer och dess miljö påverkar eCB signalering, inte minst beträffande 2-AG, dess syntes och nedbrytning.
**Key abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AEA</td>
<td><em>N</em>-arachidonoylethanolamine, anandamide</td>
</tr>
<tr>
<td>2-AG</td>
<td>2-arachidonoylglycerol</td>
</tr>
<tr>
<td>eCB/s</td>
<td>Endocannabinoid/s</td>
</tr>
<tr>
<td>CB</td>
<td>Cannabinoid</td>
</tr>
<tr>
<td>URB597</td>
<td>Synthetic FAAH inhibitor</td>
</tr>
<tr>
<td>FAAH</td>
<td>Fatty acid amide hydrolase, catabolic enzyme</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase, prostaglandin-endoperoxide synthase</td>
</tr>
<tr>
<td>NAE</td>
<td><em>N</em>-acylethanolamine</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>HAS</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum albumin</td>
</tr>
<tr>
<td>NAAA</td>
<td><em>N</em>-acylethanolamine-hydrolysing acid amidase, catabolic enzyme</td>
</tr>
<tr>
<td>MAGL</td>
<td>Monoacylglycerol lipase, catabolic enzyme</td>
</tr>
<tr>
<td>DAGLα/β</td>
<td>Diacylglycerol lipase alpha/beta, synthesising enzyme</td>
</tr>
<tr>
<td>NAPE-PLD</td>
<td><em>N</em>-acyl phosphatidylethanolamine specific phospholipase D, synthesising enzyme</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumour necrosis factor alpha</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>IC50</td>
<td>Inhibitory dose rendering half max inhibition</td>
</tr>
<tr>
<td>NSAID</td>
<td>Nonsteroidal anti-inflammatory drugs</td>
</tr>
<tr>
<td>THC</td>
<td>Phytocannabinoid, CB receptor agonist</td>
</tr>
<tr>
<td>N</td>
<td>Number of separate experiments</td>
</tr>
<tr>
<td>Ct</td>
<td>Cycle threshold</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide, used as solvent</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol, used as solvent</td>
</tr>
</tbody>
</table>
Introduction

The endogenous cannabinoid (endocannabinoid, eCB) system is an evolutionarily conserved signaling system consisting of two receptors called cannabinoid receptor 1 (CB$_1$) and CB$_2$, their endogenous ligands and the enzymes that synthesise and degrade these molecules. But a broader definition of the eCB system can also include any other endogenous molecules that bind the CB-receptors and other receptor to which these ligands bind. The characterisation of the eCB system started with the cloning of a receptor in rat and human brain responsible for binding natural and synthetic cannabinoids such as Δ$_9$-tetrahydrocannabinol (THC) the main psychoactive compound in cannabis (Devane et al., 1988; Matsuda et al., 1990, Gérard et al., 1990). Soon after, a second receptor was found (Munro et al., 1993). Today, the eCB system is known as a diverse signaling system with a wide variety of biological effects. Many of the processes are homeostatic in their function, thus eCBs are synthesised when and where they are needed and quickly internalised and degraded. eCBs are involved in control of appetite, pain, inflammation, reproduction and tumour progression among other functions, although their exact role and contribution in several of these processes is yet to be established (Ligresti et al., 2016). Many voices in society are raising their opinions and debating around the potential use of plant-derived cannabinoids as therapeutic substances, while at the same time, the adverse health effects of synthetic “spice”-like cannabinoids are becoming apparent. Thus, the eCB system continues to be a “smoking hot” area of biology. Below follows a presentation of (for this thesis) essential knowledge about the eCB system. The studies presented in this thesis focus upon $N$-arachidonoylethanolamine (AEA, anandamide), thus emphasis of the introduction is put upon AEA.

Endocannabinoids (eCBs)

The two main endogenous ligands for the CB receptors are AEA and 2-arachidonoylglycerol (2-AG). AEA belongs to the N-acylethanolamine (NAE) family together with palmitoylethanolamide (PEA), oleoylethanolamide (OEA) and stearoylethanolamide (SEA) to mention a few. NAEs are endogenous lipid mediators consisting of a variable fatty acid linked to an ethanolamine (EA) by an
amide bond (Appendix). AEA is the only major NAE to bind to and activate the CB receptor. In mammalian brains and plasma, AEA comprise about 1% of the total NAE (Schmid et al., 1995). On the other hand, in human seminal fluid and the prostate, AEA levels are higher (Madaan et al., 2000; Schmid et al., 2002).

AEA and 2-AG (discussed below) are produced in the lipid membranes of the cell from membrane-lipid precursors with a common arachidonic acid moiety (Devane et al., 1988; Matsuda et al., 1990). The synthesis of eCBs is stimulated by increased Ca\(^{2+}\) concentration as a consequence of depolarization, damage or receptor stimulation (Pitler and Alger, 1992; Hansen et al., 1997; Wilson and Nicoll, 2001). The major pathway for generation of AEA is from N-arachidonoyl phosphatidylethanolamine (NAPE) via the action of cytosolic phospholipase A2 (cPLA2) (Ogura et al., 2016) and Ca\(^{2+}\) sensitive NAPE-selective phospholipase D (NAPE-PLD).

A second pathway to generate AEA, characterized in a macrophage like cell-line generates AEA via the action of phospholipase-C, serine hydrolases and phosphatase N22 (Liu et al., 2006; Simon and Cravatt, 2006). This pathway may contribute substantially to AEA generation, considering the unchanged levels of NAEs such as AEA in NAPE-PLD knockout mice (Leung et al., 2006; Liu et al., 2006; Simon and Cravatt, 2006, 2010). In macrophages, LPS stimulate AEA generation via phospholipase C and phosphatase N22 at the same time as the expression of NAPE-PLD is reduced. Thus, implying that this second pathway is dominant during these conditions, at least in macrophages (Liu et al., 2006).

2-AG belongs to a group of lipid mediators named monoacylglycerols and is mainly synthesised from phosphatidylinositol. Phosphatidylinositol is converted by phospholipid hydrolysis and Ca\(^{2+}\) dependent diacylglycerol lipases-α or -β (DAGL-α or -β) (Bisogno et al., 2003). Knockout of DAGL-α and -β in mice generates an 80% decrease of 2-AG in brain and spinal cord and a 90% reduction in the liver (Gao et al., 2010). 2-AG can also be generated via phosphatidylinositol by Ca\(^{2+}\) independent phospholipase A and selective phospholipase C (Ueda et al., 1993). Other monoacylglycerols, such as 2-palmitoylglycerol and 2-linoleoylglycerol, have been identified. These do not interact directly with CB receptors, but can potentiate the
effects of 2-AG, an ability referred to as an “entourage” effect by Ben-Shabat et al., (1998).

**Cannabinoid receptors**

AEA, primarily act as a partial agonist at the CB receptors (Showalter et al., 1996; Gonsoirak et al., 2000). 2-AG binds CB$_2$ with similar affinity as AEA, however 2-AG acts as a full agonist at both CB$_1$ and CB$_2$ receptors (Munro et al., 1993; Gonsoirak et al., 2000).

The CB receptors are found distributed all over the body, although they each have a defined niche. CB$_1$ is mainly found in the central nervous system (CNS), located in the presynaptic membranes of GABAergic and glutaminergic neurons and in astrocytes (Navarrete and Araque, 2008; Marsicano and Lutz, 1999). The expression is higher in cortex, striatum, cerebellum, amygdala and hippocampus, regions important for memory consolidation, anxiety, fear and nociception (Matsuda et al., 1990; Herkenham et al., 1990). However, CB$_1$ receptors are also found peripherally both in neuronal and non-neuronal tissue. In the human prostate gland, for example, functionally active CB$_1$ receptors are found in the epithelial cells, but not the stroma (Ruiz-Llorente et al., 2003). In the rat prostate, CB receptor stimulation inhibits contractions of the gland in response to electrical field stimulation secondarily to release of a yet unidentified inhibitory factor acting upon prejunctional nerve endings (Tokanovic et al., 2007).

Although the CB$_2$ receptor is also found in the CNS its expression is much more established in peripheral tissue, more specifically in immunological and hematopoietic cells and organs such as the tonsils, spleen and the thymus (Munro et al., 1993; Galiègue et al., 1995; Van Sickle et al., 2005; Gong et al., 2006). CB$_2$ is especially abundant in natural killer cells, monocytes and in B-lymphocytes but significant expression is also found in neutrophils and T-cells (Munro et al., 1993; Galiègue et al., 1995; Graham et al., 2010).

Functional CB$_1$ receptors are located both in the cellular membrane and in intracellular vesicles (Rozenfeld and Lakshmi, 2008). AEA and 2-AG are both lipophilic compounds and they interact with the intramembranous accessible parts
of the trans-membranous helix of the CB₁ receptor (Lynch and Reggio, 2006). The receptors are G-protein coupled receptors, spanning the membrane seven times with the C-terminal in the intracellular lumen. The CB receptors may signal alone or in complexes as homo/heterodimers (Glass and Felder, 1997; Wager-Miller et al., 2002; Coke et al., 2016). Binding of a ligand in the membrane passing region induce a conformational change causing the C-terminal to interact with mainly the Gᵢ subtype but in special settings (predominantly CB₁) also Gₛ subtypes of G-proteins (Howlett and Fleming, 1984; Howlett et al., 1985; Glass and Felder, 1997). Gᵢ proteins inhibit adenylate cyclase leading to reduced cyclic AMP (cAMP) generation and an overall inhibitory effect upon the cell, Gₛ has the opposite effect (Howlett et al., 1985; Devane et al., 1988; Slipetz et al., 1995). For example, decreased cAMP levels reduce the activity of cAMP-dependent protein kinase A (PKA) coupled to decreased phosphorylation of potassium channels and consequently a hyperpolarization (Childers and Deadwyler, 1996). Additionally, CB₁ receptor activities secondarily modulate the activity of a variety of ion channels, activating inwardly rectifying K⁺-channels (Mackie et al., 1995), inhibiting L-type Ca²⁺ channels and N- and Q-type Na⁺ channels (Pan et al., 1996; Gebremedhin et al., 1999).

**Other receptors**

The bradycardic and hypotensive effects mediated by CB agonists are completely blocked by CB₁ knockout or treatment with the CB₁ receptor inverse agonist rimonabant (Járai et al., 1999; Ledent et al., 1999). On the other hand, motor-inhibition, analgesic and anti-inflammatory effects mediated by AEA persist in CB₁ knockout mice and after blockade by CB₂ antagonists, indicating that additional targets are operative (Járai et al., 1999; Ledent et al., 1999; Di Marzo et al., 2000; Lichtman et al., 2004b). The most well established targets are considered below.

AEA (low intrinsic activity agonist) and 2-AG interact with transient receptor potential vanilloid 1 (TPVR1) modulating pain in both the CNS and in peripheral tissue (Zygmunt et al., 1999; 2013; Ross et al., 2001). TRPV1 is a nonselective cation channel, predominantly located in the cell membrane of small diameter nociceptive sensory neurons. TRPV1 is also found centrally in dorsal root ganglions and in peripheral organs such as kidney, liver, pancreas, spleen and testis of humans as
well as in the brain (Premkumar and Sikand, 2008). The ion channel is activated by capsaicin, heat and acidic pH producing painful sensations and mediating vasodilation of small arteries (Caterina et al., 1997; Tominaga et al., 1998; Zygunt et al., 1999). In the brain, TRPV1 is associated with modulation of anxiety-related behaviors, conditioned fear and long-term potentiation in the hippocampus (Marsch et al., 2007). TRPV1 is desensitised by capsaicin and sensitised by protons (low pH). Sensitisation is coupled to hyper-algesia, and capsaicin is used to treat neuropathic pain by desensitisation of the receptor (Tominaka et al., 1998; De Petrocellis et al., 2001; O’Neil and Brown, 2003; Bhave et al., 2003; Jung et al., 2004; Premkumar et al., 2004).

AEA also binds and activates peroxisome proliferator activated receptor (PPAR) -α and –γ. (PPAR-α and –γ). PPARs are members of the nuclear receptor superfamily and act as transcription factors forming heterodimers with the retinoic acid receptor. AEA association with PPAR-α and –γ mediate anti-inflammatory effects and differentiation of fibroblasts to adipocytes respectively (Bouboula et al., 2005) 2-AG interacts with PPAR-γ reducing neuro-inflammation and amyloid-β formation (Zhang et al., 2014). 2-AG and 2-AG metabolites also bind PPAR-α/β (Kozak et al., 2000; 2002).

In addition, AEA also blocks acid-sensitive background K+ channels called TASK-1 at low concentrations and partly block TASK-3 at mediate high concentrations independently of CB-receptors, phosphorylation, Ca2+ and G-proteins. Blockage facilitates depolarization and excitability playing a role in setting the membrane resting potential (Maingret et al., 2000).

Further, AEA is suggested to interact with several orphan G-protein coupled receptors based on receptor family relationships and structure similarities (reviewed by Morales and Reggio, 2017). AEA and 2-AG have shown to bind the orphan G-protein coupled receptor 55 (GPR55) at concentrations similar to CB, and activate systems which involve the receptor (Ryberg et al., 2007), although the identification of GPR55 as a potential CB receptor is controversial.

As indicated by the diversity of proposed and established receptors for AEA, the eCB system is an expanding, and highly diverse signaling system.
**eCB uptake**

The uptake-mechanism of AEA is temperature-dependent, concentration-dependent and saturable (Di Marzo et al., 1994; Hillard et al., 1997). Given that eCBs are highly lipophilic compounds, they can pass the plasma lipid membrane by simple diffusion and there is an ongoing debate as to whether such diffusion and/or carrier-mediated transport across the plasma membrane by a hitherto unidentified transport protein is the primary mechanism for the cellular uptake of AEA (for reviews, see Fowler, 2013a; Nicolussi and Gertsch, 2015). Once inside the cell AEA binds to carrier proteins such as fatty acid binding proteins (FABP), heat-shock protein 70 (Hsp70) and albumin (Kaczocha et al., 2009; Oddi et al., 2009). A catalytically inactive splice variant of FAAH named FAAH-like AEA transporter (FLAT) has also been presented as an AEA carrier protein (Fu et al., 2011). However, the existence of FLAT is questioned, as other research groups have failed to find expression of such a FAAH splice variant (Leung et al., 2013).

Intracellular protein bound AEA is shuttled to receptors or enzymes for breakdown. At least a part of the uptake of AEA is driven by a concentration gradient. Thus, a continuous degradation of the internalised eCB keeps the gradient active. This is supported by the decreased (but not totally eliminated) uptake of AEA in the presence of fatty acid amide hydrolase (FAAH) inhibitors, and the increase in uptake produced by transfection of cells with FAAH (Day et al., 2001; Deutsch et al., 2001; Glaser et al., 2003). Exogenously added AEA is also efficiently and continuously hydrolysed by vesicles containing internally trapped FAAH but devoid of any transport protein or additional metabolising enzymes (Kaczocha et al., 2012a).

The uptake of 2-AG is more sparsely investigated. The uptake is not primarily regulated by 2-AG hydrolysis by MAGL in C6 (rat glioma) RBL2H3 (rat basophilic cells), PC3 (human prostate) or AT-1 (rat prostate) cells but may exert a prominent role in other cell types as was found in Neuro-2a (mouse neuroblastoma) cells (Beltramo and Piomelli, 2000; Fowler and Ghafouri, 2008; Chicca et al., 2012). The uptake-mechanism resembles the mechanism for AEA in that the uptake of 2-AG is inhibited by OMDM-2 (suggested to bind FABP5) and the eCB uptake inhibitor UCM707 (Chicca et al., 2012; Kaczocha et al., 2012b), both of which were originally
designed as AEA uptake inhibitors (López-Rodríguez et al., 2003; Ortar et al., 2003). Guineensine, a component of black pepper that binds the proposed FLAT also inhibits the uptake of both AEA and 2-AG (Nicolussi et al., 2014). Thus, AEA and 2-AG compete for binding to transport proteins but the factor keeping the concentration gradient active for 2-AG is unknown (Chicca et al., 2012).

**Degradation of eCBs**

The key pathways responsible for eCB degradation are summarized in Figure 1.

AEA and 2-AG are certainly the most studied eCBs and they both exert effects via CB and TRPV1 receptors. However the pathways responsible for their synthesis and degradation (summarized in Figure 1) is separated making the control of each eCB independent from the other.

**FAAH**

FAAH is the main enzyme responsible for degradation of AEA. It has a wide substrate-specificity, and hydrolyses related NAEs and other compounds, such as N-acylamines and N-acyltaurines (Cravatt et al., 1996; 2001; Wei et al., 2006; Appendix). FAAH expression is high in the brain areas: cerebellum, hippocampus and cortex, also rich in CB1 receptors (Matsuda et al., 1990; Herkenham et al., 1990; Egertová et al., 1998). Outside of the CNS, FAAH is predominantly found in the testis, prostate, kidney, brain, spinal cord, ileum, stomach, lung and the liver (Engeli et al., 2005).

FAAH is an intracellular amidase serine-hydrolase presented as a dimer that was first isolated and characterized from rat liver plasma membranes (Cravatt et al., 1996; 2001). FAAH is found in the membrane of the endoplasmic reticulum and the mitochondria with the active site facing the cytosol (Cravatt et al., 2001; Wie et al., 2010). As described in section “eCB uptake” FAAH drives the uptake of AEA by mediating its degradation, thus sustaining a gradient driven uptake mechanism.
The enzyme mainly responsible for AEA degradation is FAAH, secondly NAAA hydrolys all NAEs with preference for PEA. 2-AG is mainly hydrolysed by MAGL, secondly ABHD6 and ABHD12 may also contribute. Both AEA and 2-AG are bioactivated by COX-2, the products are prostaglandin (PG) derivatives with biological activities of their own. Included in the figure is also the parallel synthesis pathway of PGs from arachidonic acid (AA). Other abbreviations: EA, ethanolamide; G, glycerol; TXA₂, thromboxane A₂. Lipooxygenase and CYP-catalysed pathways for eCB degradation have also been described (see the main text).

The global structure of FAAH has three cavities and simultaneous access to both the cytosolic inner compartment and the lipid membrane. Two channels are proposed to be entry and binding ports for substrate while the third channel is proposed to allow release of products and access to water for the hydrolytic reaction (Bracey et al., 2002). The hydrolysis of NAEs by FAAH generates a free fatty acid and an ethanolamide.

The activity of FAAH is pH dependent, with an optimum at pH 9 (Schmid et al., 1985; Omeir et al., 1995; Ueda et al., 1995a). The intracellular pH of a cell generally
ranges from 4.7 in the lysosome to 7.2 in the cytoplasm and pH 8 in the mitochondria (Casey et al., 2010). In solid tumours, the extracellular pH may decrease considerably (6.15-7.4), whereas the intracellular pH remains fairly well controlled in the range of 7.0-7.2 (Vaupel et al., 1989). Extracellular pH is also found to decrease in inflammation (Häbler et al., 1929; Punnia-Moorthy, 1987) and there is evidence that intracellular pH can also decrease in neuro-inflammation induced by the bacterial toxin lipopolysaccharide (LPS) (Tyrtysnhaia et al., 2016). In such circumstances, FAAH may function less than optimally.

Pharmacological inhibition or genetic deletion of FAAH increases NAE levels and reduce pain perception, inflammation, oedema and anxiety in a partly CB receptor mediated way but does not affect cognitive functions as CB agonist do (Cravatt et al., 2001; Kathuria et al., 2003; Lichtman et al., 2004a; 2004b; Naidu et al., 2009).

FAAH is inhibited by general serine hydrolase inhibitors. Phenylmethyl-sulfonyl-flouride (PMSF) and methylarachidonoyl-flouro-phosphonate (MAFP) were the first defined FAAH inhibitors (Deutsch and Chin, 1993; Compton and Martin, 1997; Deutsch et al., 1997). PMSF and MAFP belong to the flouro-sulphonate and flourophosphionate group of inhibitors respectively and are both unselective, irreversible inhibitors of FAAH. Oleoyl trifloromethyl-ketone (OTMK, trifloromethyl ketones) and 7-phenyl-1-(5-pyridin-2-yl-1,3-oxazol-2-yl)heptan-1-one (OL-135, alpha-ketoheterocycle) are reversible FAAH inhibitors with $K_i$ values of 82 nM and 4.7 nM respectively (Patricelli et al., 1998; Boger et al., 1999; 2005). OL-135 is FAAH selective and produces in vivo analgesia in mice (Lichtman et al., 2004a).

URB597 ((3'-(aminocarbonyl) [1,1'-biphenyl]-3-yl) -cyclohexylcarbamate) (Kathuria et al., 2003) was the first selective FAAH inhibitor described and is the inhibitor mainly used in this thesis. This carbamate compound is a potent, irreversible inhibitor of FAAH. URB597 is selective for FAAH in the brain ($K_i$ 2 µM) however it inhibits several carboxylesterases in the liver at higher concentrations ($IC_{50}$ values ≥ 1 µM) (Kathuria et al., 2003; Lichtman et al., 2004a; Alexander and Cravatt, 2005; Wei et al., 2006). In rat models, irreversible URB597 decrease anxiety behavior, pain and inflammation (Kathuria et al., 2003; Holt et al., 2005). More recently described compounds are N-3-pyridinyl-4-[[3-[5-(trifluoromethyl)-2-pyridinyl]-oxy]phenyl]methyl]-1-piperidine-carboxamide (PF-3845, $K_i$ 0.23 µM) and N-3-
pyridazinyl-4-[[3-[5-(trifluoro-methyl)-2-pyridinyl]oxy]phenyl]-methylene]-1-piperidinone-carboxamide (PF-04457845, Pfizer). These are irreversible/slowly reversible inhibitors of the piperazine urea family, selective for FAAH in both the brain and the liver (Ahn et al., 2007; 2009; Johnson et al., 2011). Several FAAH inhibitors have been developed for therapeutic treatment of pain, however none have yet passed the clinical trials (Appendix).

Acidic profens within the non-steroidal anti-inflammatory (NSAID) group of drugs such as ibuprofen (IC\textsubscript{50} 270 \textmu M) and flurbiprofen (IC\textsubscript{50} 50 \textmu M) also inhibit FAAH, although with low potencies (Fowler et al., 1997; 1999; Holt and Fowler, 2003). Interestingly, the potencies increase with lower pH (Holt et al., 2001), possibly partly due to efficacy of the non-ionized, rather than ionized form of the molecules at FAAH.

**MAGL, ABHD6, ABHD12**

The main enzyme hydrolysing 2-AG, responsible for roughly 85% of the degradation in the brain is monoacylglycerol lipase (MAGL) (Dinh et al., 2002; 2004; Blankman et al., 2007). MAGL is a dimer and a \( \alpha/\beta \) hydrolase (serine hydrolase) found both associated to the membrane and free in cytoplasm. MAGL hydrolyses monoacylglycerols and differs from its closest homologues in the substrate binding structure and in the cap-domain that covers the active site (Labar et al., 2010). The cap-domain consists of a helix flanked by two loops making the cap flexible (Bertrand et al., 2010). The cap domain helix is lipophilic, allowing MAGL to dock to lipid membranes and reach its lipophilic substrates. The active site is found in the bottom of a large cone formed channel, partly lined with hydrophobic amino acids at the top. The active site, a small cavity and a narrow channel nearby are hydrophilic to allow binding and release of the glycerol (Bertrand et al., 2010; Labar et al., 2010).

The remaining 2-AG not accounted for by MAGL can be roughly ascribed to FAAH, \( \alpha/\beta \)-hydrolase domain containing-6 and -12 (ABHD6 and ABHD12), although the importance of the hydrolytic enzymes depends upon their relative expression, which can vary between cells and organs (Fowler, 2012). ABHD6 and -12 are integral membrane enzymes positioned with their active site facing the extracellular and the
intracellular milieu respectively (Blankman et al., 2007). Degradation of 2-AG by MAGL and ABHD6 and -12 produces a free fatty acid and a glycerol.

**COX-2**

COX-2 and its isoenzyme COX-1 are key enzymes in the production of prostaglandins, thromboxanes and prostacyclins. The COX enzymes are homodimers and are bound to the membrane of the endoplasmic reticulum. In comparison with COX-1 that is constitutively active, COX-2 expression is essentially induced by inflammatory cytokines, growth factors and tumour promoting factors. Thus, in addition to the common domains for membrane-association, epidermal growth factor (EGF) like-domain and the catalytic domain, COX-2 contains a degradation-controlling subunit. The catalytic domain contains two active sites oriented on opposite sides of a heme prosthetic group. The sites catalyse one of two reactions each, cyclooxygenation and peroxidase reactions. COX-2 has a second pocket in its active site channel created by a few amino acid substitutions compared to COX-1 (Vecchio and Malkowski, 2011; Picot et al., 1994). The pocket allows COX-2 to catalyse reactions of AEA and 2-AG in addition of arachidonic acid. COX-2 oxygenates 2-AG equally effective, and AEA at a rate 60-85% of arachidonic acid (Yu et al., 1997).

The products of COX-2 catalysed reactions of eCBs are prostaglandin ethanolamides (from AEA, also known as prostamides) and prostaglandin glycerol esters (from 2-AG). The biological role of these signaling molecules is separate from the eCBs and not fully known. However, selected compounds are implicated in pain and inflammation, suggestively exerting both pro and anti-inflammatory effects (reviewed by Alhouayek and Muccioli, 2014). For example, bimatoprost (prostamide analogue of PGF2α) is a pharmaceutical for glaucoma and high ocular pressure, reducing the intraocular pressure by increasing the ocular outflow (Brubaker, 2001).

NSAIDs are the most recognized and publicly known inhibitors of the COX-enzymes, with varying isoenzyme-specificity within the large group of drugs. A major problem with the NSAIDs as therapeutic drugs is the high association with gastro-intestinal adverse events such as bleeding. This adverse reaction occurs due to reduced production of homeostatic prostaglandins (predominantly PGE₂) in the
gastric mucosa (Takeuchi, 2012). Coxibs are COX-2 selective inhibitors developed to circumvent the adverse events associated with the inhibition of both COX-isofoms (Peskar, 2001). However, the coxibs turned out to be associated with an increased risk of heart disease (Bing and Lomnicka, 2002), an issue that is also relevant for the NSAIDs.

**Other metabolic pathways**

AEA and the other members of the NAE family are hydrolysed by N-acylethanolamine-hydrolysing acid amidase (NAAA). NAAA is highly expressed in the lysosomes of macrophages and other immune cells where it is activated by autoproteolytic cleavage at pH 5 (Appendix). NAAA prefer saturated NAEs, predominantly PEA. Thus AEA is hydrolysed by NAAA only to a minor extent (Tsuboi et al., 2005). NAAA inhibitors are emerging for therapeutic intentions to treat pain and inflammation (reviewed by Bottemanne et al., 2018).

eCBs are also substrates for lipoxygenase (LOX) and cytochrome P450 (CYP) enzymes. 12-LOX and 15-LOX from rat, porcine and soybean respectively oxidize AEA producing mainly 12-hydroperoxy-5,8,10,14-eicosatetraenoyl-ethanolamide (12-HpETE-EA) and 15-hydroperoxy-5,8,11,13-eicosatetraenoyl-ethanolamide (15-HpETE-EA). The LOX-12 product is active at the CB receptors and 15-HpETE-EA inhibits electrically-evoked contractions of mouse vas deferens (IC$_{50}$: 0.63 µM (Ueda et al., 1995b; Hampson et al., 1995). 2-AG is oxidized by porcine 12-LOX and 15-LOX (Moody et al., 2001; Kozak et al., 2002).

Cytochrome P 450 (CYP450) enzymes are a large group of heme-containing oxygenases responsible for degradation of a variety of endogenous (steroids, fatty acids, neuro-transmitters) and exogenous substances (drugs, prodrugs, drug metabolites, toxins). Primarily CYP isofoms 4F2 hydroxylates AEA to form 20-hydroxyl-eicosatetraenoic acid ethanolamides (20-HETE-EA) in the human kidney and liver. AEA is also epoxidized to 14,15- and 5,6-epoxyeicosatrienoic ethanolamide (14,15- and 5,6-EET-EAs) by CYP 3A4 in the liver, CYP 2D6 and the orphan enzyme 4X1 in the brain (Snider et al., 2007; Stark et al., 2008). 5,6-EET ethanolamide is found to be a potent and selective agonist of CB1 possibly having a role in the context of immune function (Snider et al., 2008).
The biological role of eCBs

The eCBs are involved in a wide variety of biological effects via activation of the CB receptors as well as other receptors (Ligresti et al., 2016). Examples of relevance to the present thesis are discussed below:

**eCBs and pain**

In animal models of inflammatory and neuropathic pain, activation of CB receptors produces analgesia by central, spinal and peripheral mechanisms (Lichtman and Martin, 1991). Cannabinoids suppress nociceptive neuro-transmission with potencies and efficacies equal to morphine (Walker et al., 1999). Locally administrated AEA attenuates formalin induced peripheral pain via CB₁ on nociceptors and inhibit hyperalgesia in rat paw withdrawal tests of carrageenan induced pain and capsaicin induced oedema (Calignano et al., 1998; Richardson et al., 1998). In addition, selective deletion of FAAH in peripheral organs of mice generates an anti-nociceptive phenotype (Cravatt et al., 2004b). In total FAAH knockout mice the reduced nociception is mediated by intensified AEA signaling (Cravatt et al., 2001). Indeed, inhibition of FAAH via URB597 and OL-135 induce analgesia (Lichtman et al., 2004a; Krustev et al., 2014). Peripheral neuronal injury induces CB₂ receptor expression, and spinal administration of CB₂ agonists decrease the neuronal hypersensitivity induced by spinal nerve ligation (Ibrahim et al., 2003; Wotherspoon et al., 2005)

Centrally in the nervous system eCBs activate rostral ventromedial medulla (RVM) circuits that projects descending pain inhibitory signals to the dorsal horn in a similar way as opioids mediate analgesia. eCBs may also control the basal nociceptive threshold via modulation of activity in the RVM (Meng et al., 1998). The role of the eCB system in tonical nociceptive regulation is supported by the increased hyperalgesia produced by intrathecal SR141716A blockage of CB₁ receptors in the hot-plate test (Richardson et al., 1997).

As described in section “Other receptors”, AEA is an agonist of low efficacy to TRPV1 (Ross et al., 2001). CB₁ and TRPV1 are frequently coexpressed in neurons along the nociceptive signaling pathway. When coexpressed, CB₁ agonists
suppressed TRPV1 activation increasing the activation threshold level (Yang et al., 2013). Increased levels of AEA in an arthritic rat model suppress hypersensitivity of afferent sensory neurons and elevated pain thresholds via CB1 and TRPV1 channels (Starowicz and Przewlocka, 2012). TRPV1 knockout in an arthritis animal model show that TRPV1 mediate both pain and inflammation (Szabo et al., 2005).

**eCBs in the immune system**

The eCB system produces anti-inflammatory effects primarily via CB2 receptor signaling. The expression of CB2 is higher in natural killer cells, monocytes and in B-lymphocytes than in neutrophils and T-cells (Munro et al., 1993; Galiègue et al., 1995; Graham et al., 2010).

CB2 receptors are expressed in the extending front of active migrating microglial cells (Walter et al., 2003). The synthetic non-selective CB receptor agonist CP55-940 inhibit spontaneous macrophage migration and chemotaxis induced by methionyl-leucine-phenylalanine (fMLP) *in vitro* by a mechanism primarily dependent upon CB2 receptor (Sacerdote et al., 2000). CB2 agonists decrease monocyte/macrophage differentiation, migration and decrease the expression and secretion of pro-inflammatory factors such as interleukin-12 (IL-12), tumour necrosis factor α (TNFα) and genes associated with TNFα and LPS (Persidsky et al., 2015; Roth et al., 2015). In addition, CB2 receptor activation induces apoptosis in B and T cells (Lombard et al., 2007).

CB2 receptor abundance increases with activation of T-cells, and stimulation of these receptors by THC, JWH-015 and O-1966 inhibits T-cell proliferation and activation and instead promotes a T-regulatory phenotype (Klein et al., 1985; Robinson et al., 2013; 2015). In a CB2 knockout host *versus* graft mouse model, dendritic cells secrete more IL-12 and IL-23 cytokines that stimulate T-helper cells. These T-helper cells showed an enhanced capacity to differentiate into interferon (IFN)-γ or IL-17-producing effector cells. In the same CB2 knockout model, graft rejection was accelerated compared to wild-type and the secretion of pro-inflammatory cytokines IL-1β, IL-6 and TNFα as well as transforming growth factor-β1 was increased in dendritic cells upon activation by LPS or by CpG motifs (Robinson et al., 2013; 2015).
In arthritis, inflammation and infiltration of immune cells leads to hyper-sensitivity and release of inflammatory factors. Malfait et al. (2000) have shown that eCBs can block progression of joint inflammation in rodent models of arthritis. The protective CB\(_2\) mediated effects include the suppressed secretion of pro-inflammatory cytokines and damaging proteinases as well as regulation of immune cell adhesion and migration (Burston et al., 2013).

In vivo studies by Krustev et al. (2014) reported that FAAH inhibition with compound URB597 elevates tissue concentrations of AEA by inhibiting local eCB degradation and dampen inflammatory pain in rodent models of osteo-arthritis. In a similar study, URB597 suppressed inflammatory hyperemia in a mouse model of acute arthritis (Krustev et al., 2014). In another rat model, URB597 decrease anxiety behavior, pain and inflammation with associated oedema (Kathuria et al., 2003).

LPS is a major activator of macrophages and is an efficacious inflammatory agent that induces hypotension and tachycardia. In vitro experiments show an increase in AEA levels in macrophages and 2-AG levels in platelets after treatment with LPS (Varga et al., 1998). In a rat model a hypotensive effect was induced not only via LPS but also via LPS stimulated platelets and macrophages. The hypotensive effect of LPS was blocked CB\(_1\) antagonism (SR141716A) indicating that eCBs in platelet and macrophages play a role in the systemic effects seen by LPS (Varga et al. 1998). In mouse macrophage RAW264.7 cells, AEA synthesis is indeed stimulated upon exposure to LPS but also upon exposure to THC, AEA itself and indomethacin (Pestonjamasp and Burstein, 1998). In contrary to these findings LPS decrease the expression of AEA synthesising enzyme NAPE-PLD and induce or leave the expression of FAAH unchanged (Murakami et al., 2007; Liu et al., 2003; 2006). Cellular levels of 2-AG are unaffected by LPS (Liu et al., 2003).

**FAAH inhibitors in pain and inflammation: the case for “dual target” drugs**

Phytocannabinoids produce some therapeutic effects in pain conditions, but this should be considered with respect to potential untoward side effects upon executive functions (reviewed by Cohen and Weinstein, 2018). A meta-analysis of studies investigating herbal cannabinoids for treatment of chronic pain conditions showed a
small reduction in pain intensity over a treatment period of 25 days (standardised mean difference was -0.61 on the visual analogue scale (VAS)). However, the risk of CNS related adverse events was high, OR > 3 for euphoria, mood disturbance, speech disturbance, blurred vision, impaired memory, numbness, ataxia, attention disturbances and disconnected thoughts (Martín-Sánchez et al., 2009). This puts a limitation on the therapeutic usefulness of such compounds.

Instead, increasing the eCB signaling by indirectly raising the endogenous eCB levels through modulation of its degradation is explored as a pharmacological strategy. Pharmacological inhibition or genetic deletion of FAAH increases NAE levels and produce potentially beneficial effects in animal models of many pain conditions (e.g. neuropathic, inflammatory, cancer pain) and of inflammation, without producing changes in cognitive functions such as are found with CB₁ receptor agonists (see e.g. Cravatt et al., 2001; Lichtman et al., 2004a; 2004b; Holt et al., 2005; Khasabova et al., 2008; Naidu et al., 2009; Krustev et al., 2014). However, clinical trials with FAAH inhibitors for the treatment of pain have given disappointing results, so something has been “lost in translation” (Appendix). Two explanations for this arise from the multiplicity of AEA targets and catabolic pathways. As described in the “Other receptors” section above, AEA is a low efficacy agonist of TRPV₁ (chemically and thermally activated) (Ross et al., 2001). CB₁ and TRPV₁ are frequently coexpressed in neurons along the nociceptive signaling pathway. In cultured dorsal root ganglia neurons treated with inflammatory mediators, the efficacy of AEA towards TRPV₁ receptors increases, suggestive of a switch from eCB to endovanilloid signalling (Singh Tahim et al., 2005). However, when coexpressed, CB₁ agonists suppressed TRPV₁ activation, increased the activation threshold level and reduced the inflammatory response in mouse corneal tissue (Yang et al., 2013). Further, increased levels of AEA in an arthritic rat model suppress hypersensitivity of afferent sensory neurons and elevated the pain thresholds via CB₁ and TRPV₁ channels (Starowicz and Przewlocka, 2012).

Secondly, increased levels of AEA could be handled by other enzyme whose role in AEA degradation is potentiated in the absence of FAAH. A strategy to circumvent this problem and increase the likelihood of elevated AEA levels is dual target inhibitors, inhibitors with affinity and inhibitory activity towards several targets. A possible dual-target candidate is COX-2, given that it is induced at sites of
inflammation and pain. FAAH (and MAGL) inhibition acts synergistically with NSAIDs in experimental pain models (Naidu et al., 2009; Crowe et al., 2015). Additionally, genetic deletion or pharmacological inhibition of FAAH or inhibition of MAGL reduces the gastro-intestinal damage produced by NSAIDs (Naidu et al., 2009; Sasso et al., 2012; Crowe and Kinsey, 2017). In 2003, Cocco et al. reported that an analogue of ibuprofen, ibu-AM5, showed analgesic efficacy in an animal model of visceral pain, but did not produce gastro-intestinal disturbances. This compound was later shown to be 2-3 orders of magnitude more potent than ibuprofen as an inhibitor of FAAH. Ibu-AM5 also inhibits COX isoforms, in fact with selectivity towards the inhibition of COX-2 mediated AEA oxygenation over arachidonic acid oxygenation (Holt et al., 2007; Fowler et al., 2013b). A similar pattern (this time measured with 2-AG rather than AEA as COX-2 substrate) was found for the corresponding analogue of flurbiprofen, flu-AM1 (Cipriano et al., 2013). Both compounds have chiral centers and at the start of this thesis it was not known whether the enantiomers had different potencies towards FAAH and COX-2. This is the case for profens such as ibuprofen (Holt et al., 2001; Duggan et al., 2010).

An alternative approach has been to design and thereafter optimize molecules containing structural elements of URB597 and flurbiprofen. This has led to the discovery of ARN2508, which shows efficacy in animal models of inflammation and colitis, but does not produce gastric toxicity (Sasso et al., 2015).

**eCBs in tumour development and progression**

The eCB system is implicated in the development and progression of tumours, although the picture is somewhat complicated. Early studies demonstrated that THC, CB agonists, eCBs and their stable analogues mediate potentially beneficial effects upon apoptosis, proliferation, invasion, migration and adhesion of cancer cells of prostate, breast, colorectal, lung and glial cell tumour origin (De Petrocellis et al., 1998; Galve-Roberh et al., 2000; Jacobsson et al., 2001; Mimeault et al., 2003; Nithipatikom et al., 2004; Wang et al., 2008; Olea-Herrero et al., 2009; Orellana-Serradell et al., 2015; Winkler et al., 2016), and these findings are supported by in vivo studies using xenografts. Tumour cell lines and tumour tissue often have an increased expression of CB receptors and there is some evidence of
increased tumour eCB levels, although in most studies the number of cases investigated is very low (review, see Flygare and Sander, 2008). In perhaps the most influential study, Galve-Roperh et al. (2000) inoculated rats with glioma cells (C6 cell line) to induce malignant tumours. Rats that were untreated died after 12 to 18 days, however rats receiving THC or the synthetic CB receptor agonist WIN-55,212-2 injections at the site of inoculation survived significantly longer. In a few cases the tumours were completely eradicated. Follow up in vitro experiments in C6 subclones sensitive or resistant to the apoptotic effects of THC (1 µM) indicated that its anti-tumourigenic effect was induced by CB₁ and CB₂ receptor-mediated actions upon ceramide and the Raf1/ERK signaling pathway controlling the cell cycle.

These findings are rather strange, since it would suggest that the tumours express something that is to their disadvantage. This does not seem to be the case, since a high expression of tumour CB₁ receptors is associated with a poor prognosis in pancreatic cancer (Michalski et al., 2008), prostate cancer (Chung et al., 2009), colorectal cancer (Gustafsson et al., 2011) and oesophageal squamous cell carcinoma (Hijiya et al., 2017). Pharmacologically relevant (nM as opposed to µM) concentrations of THC are often mitogenic (Nadulski et al., 2005; Fowler, 2015). Further, the in vivo effects of THC in xenograft models, which use immunocompromised animals, are not seen when syngenic immunocompetent animals are used, indeed THC is mitogenic in these models (Zhu et al., 2000; McKallip et al., 2005). Knockout of both CB receptors in mice decreases the occurrence of ultraviolet-light induced skin cancer suggesting that eCBs promote tumour formation in the skin (Zheng et al., 2008). However, the picture is not uniform: A low, rather than a high tumour CB₁ expression is associated with a poor prognosis in hepatocellular carcinoma (Xu et al., 2006), and in a mouse model (APC-mutated) of colorectal cancer deletion or inhibition of CB₁ results in an increased incidence of polyps and adenomas in the small intestine and colon (Wang et al., 2008).

In addition, enzymes involved in the synthesis and degradation of eCBs are also found to be dysregulated in cancers. Thus, enzymes FAAH, NAAA and COX-2 in prostate cancer correlate with increased disease severity and prognosis (Madaan et al., 2000; Endsley et al., 2008; Michalski et al., 2008; Chung et al., 2009; Richardsen et al., 2010; Thors et al., 2010).
Clearly the eCB system plays a role in cancer, but why and how these changes are induced is not known. Traditionally, cancer is regarded as a genetic disease arising from multiple acquired mutations in a single cell, deregulating homeostatic control of the cells. No such mutations have been found linked to the eCB system. However, Wang et al. (2008) found epigenetic silencing of the CB₁ receptor promotor with an effect upon intestinal adenoma growth in colorectal cancer. How and why this occurred is not known.

Interactions with, and adaptations to, the tumour microenvironment, including the presence of inflammation and inflammatory factors are a considerable contributor to tumour progression (Chung et al., 2005; Fernandes et al., 2015. The hypothesis of this thesis is that these factors are responsible for the dysregulated eCB system in the tumours.
Aims of the thesis

The main aim of this thesis was to explore the role of COX-2 in AEA degradation. The aims of each study are given below.

Paper I: to study the FAAH and COX inhibitory properties of the main metabolites of flurbiprofen and ibuprofen.

Paper II: to evaluate the interaction of the enantiomers of flu-AM1 and ibu-AM5 with FAAH.

Paper III: to characterize the (R)-enantiomer of flu-AM1 as a dual FAAH / substrate selective COX-2 inhibitor.

Paper IV: to investigate the effects of TNFα treatment upon the expression of eCB related genes and the functional consequence for AEA hydrolysis in human DU145 prostate cancer cells.

Paper V: to investigate the effects of IL-6 and lactic acid-induced low pH upon the expression of eCB related genes and the functional consequence for AEA hydrolysis and cell survival in rat AT-1 prostate cancer cells.
Methodological considerations

This thesis is based upon in vitro models studying the expression and activity of enzymes in isolated enzyme assays and cell cultures. In vitro models allow the characterization of individual factors and insight into the mechanisms involved in the otherwise complex biology of the cell, organism and ultimately animal. On the other hand, isolating individual events and situations that occur in the cell is a simplification of the complex and multiple associations that occur in an organism.

The detailed methodological protocols are given in each original paper. In this section the underlying basic principles, advantages and limitations of each method are described. Synthesis of molecules (II, III), computational studies (III), cloning and expression of mutant and wild-type FAAH (III) and liquid chromatography/mass spectroscopy (III/IV) were performed at other laboratories and will not be covered in this section. The laboratories involved were: synthesis of NSAID derivatives, Department of Life and Environmental Science, Unit of Pharmacological and Nutraceutical Science, University of Cagliari, Italy (co-authors Onnis, Deplano and Congiu); computational studies, Department of Pharmacy, Università degli Studi di Napoli Federico II, Napoli, Italy and the Department of Fisicoquímica and Institut de Biomedicina, facultat de Farmàcia, Universitat de Barcelona, Santa Coloma de Gramenet, Spain (co-authors Catalanotti, Morgillo, Luque, Novellino); cloning and expression of wild-type and mutant FAAH, Institute of Biostructure and Bioimaging, Naples, Italy (co-authors Pedone and Smaldone); and liquid chromatography/mass spectroscopy, Department of Chemistry, Umeå university, Umeå, Sweden (co-author Gouveia-Figueira).

Cell culture (papers II, IV, V)

Mouse leukemic RAW264.7 macrophages (passage 5-31), human DU145 prostate carcinoma (passage 15-31) and rat Dunning R3327 AT-1 prostate carcinoma (passage 18-41) cell lines were used. Cells were maintained at 37°C, 5% CO₂ in a humidified environment in appropriate cell culture medium supplemented with penicillin (100 units/ml (U/ml)) and streptomycin (100 µg/ml).
DU145 and RAW264.7 cells respond to inflammatory stimuli by increasing COX-2 production. DU145 cells were treated with 20 ng/ml TNFα in PBS with human serum albumin (HSA, final concentration 0.001%) in serum-free medium for 4 hours (h) (Subbarayan et al., 2001). RAW264.7 cells were treated with 0.1 µg/ml LPS and 100 U/ml IFNγ in 1 µM NaHPO₄ pH 8.0 with 10⁻⁵ % bovine serum albumin (BSA) (final concentration) for 24 h (Hwang et al., 1996). AT-1 cells have been used previously in the laboratory with respect to AEA uptake and hydrolysis (Thors et al., 2007), but AT-1 cells are also useful in that they are syngenic to Copenhagen rats and can thus be used in orthotopic studies in immunocompetent animals (see e.g. Halin et al., 2007). In this way, in vitro findings from our studies could form the basis for in vivo studies. AT-1 cells were treated with 25 or 100 ng/ml rat recombinant IL-6 in PBS supplemented with 0.001% BSA (final concentration) and modified Krebs-Ringer-Hepes lactate (modified KRH lactate) buffer or low serum (0 or 1%) cell culture medium pH 6.6 or 7.4 for 3 h.

**AEA hydrolysis (papers I, II, III, IV, V)**

The protocol for hydrolysis measurements is adapted from Omeir et al. (1995) by Boldrup et al. (2004). The assay utilizes the cleavage of AEA tritium labelled in the ethanolamine part ([³H]AEA) by FAAH into a lipophilic fatty acid moiety and a water-soluble [³H]-ethanolamide (Figure 1). A FAAH enzyme preparation in Krebs-Ringer-Hepes buffer pH 7.4 (KRH) supplemented with 0.1% fatty acid free BSA is incubated with the [³H]AEA at 37 °C in to allow hydrolysis. After a set period of time, the reaction is stopped by two actions, addition of absorptive charcoal and acid (low pH) and lowering of the temperature (placing reaction-tubes on ice). The lipophilic fatty acid moiety is absorbed by the charcoal and pelleted by centrifugation. Water soluble, [³H]-ethanolamide remains in the solute that is collected. The [³H]-ethanolamide content is quantified by liquid scintillation spectroscopy with quench correction. Inhibitors are added to the enzyme preparation with or without a pre-incubation phase, at 37 °C prior to the addition of [³H]AEA. Controls are samples without the enzyme preparation.
FAAH enzyme preparations were from one of four sources: cerebral brain membrane homogenates from adult Wistar or Spargue-Dawley rats (I, II, III), cerebral brain membrane homogenates from male B6CBAF1 mice (III), cell culture homogenates (II) or intact cells from culture (III, IV, V).

FAAH hydrolysis using intact cells was performed in 24-well plates. Cells were plated and treated with cytokines and/or inhibitors, when appropriate. At the time of the experiment the cells were washed with KRH supplemented with 1% BSA and plain KRH before initiation of hydrolysis according to the main protocol summarised above. Wells without cells were included in each experiment for measurement of plastic binding (background).

Different aspects of the inhibitor-enzyme interaction can be studied by small modifications to the described method. Graded dose-response curves and calculations of pI_{50} and IC_{50} are gained through hydrolysis experiments with varying concentration of inhibitor (paper I-III). The kinetics of the inhibitor-enzyme interaction is investigated by varying the concentration of inhibitor and substrate (paper II and III). Inhibitor time-dependency was investigated by varying the preincubation time where the inhibitor was allowed to interact with the enzyme prior to addition of substrate (paper II). Inhibitor cooperativity is studied by the addition of two inhibitors in varying concentrations to the reaction mix (paper II). Reversibility of binding is studied by preincubating the enzyme with the substrate. After a dilution step the hydrolysis of AEA is measured. If the compound is fully reversible, dilution will reduce the observed inhibition to the same level as that seen for the corresponding diluted concentration of inhibitor without a preincubation.
period. Dose-response relationships, kinetics, cooperativity and reversibility experiments were performed in rat/mouse brain homogenates.

How do we know that it is FAAH hydrolysis that we are measuring? FAAH is the main degradative enzyme of AEA, but COX-2, CYP450, LOX and NAAA also utilise AEA as a substrate (Cravatt et al., 2001; Yu et al., 1997; Tsuboi et al., 2005; Ueda et al., 1995b, Kozak et al., 2002; Snider et al., 2007). However, the products of oxidative metabolism by COX-2 are tritium labelled lipophilic compounds that will not be retained in the water phase of this assay. FAAH and NAAA can be distinguished by use of selective compounds such as URB597, which inhibits FAAH at nanomolar concentrations, but which does not inhibit NAAA at concentrations up to 10 µM (Sun et al., 2005). In the AEA hydrolysis assay of intact cells, 1 µM URB597 strongly abolishes the tritium detection level indicating that FAAH rather than NAAA is primarily responsible for the hydrolysis of this substrate (Figure 2 and 3). The component of AEA (and predominantly PEA) hydrolysis due to NAAA can similarly be quantitated using the inhibitor pentadecylamine (Yamano et al., 2012; Gabrielsson et al. 2017).

For the experiments in this thesis a pH of 7.4 (at room-temperature) was generally used to simulate the pH of the body. To simulate an inflammatory and tumour associated (acidic) microenvironment, FAAH activity was measured at pH 6.0 in paper I and after pretreatment with buffer adjusted to 6.6 using lactic acid in paper V. The pH of the buffers used in paper I and V was measured at the experimental temperature, 37°C.

The method used in this thesis is relatively quick and gives excellent ratios of activity to blank (Figure 2). Alternative methods to study FAAH and NAAA activity are chromatographic methods, fluorescence and colorimetric based methods (Kage et al., 2007; Patricelli and Cravatt, 2001). The fluorescent and colorimetric based methods measure the cleavage of synthetic FAAH substrate in to a product with suitable spectrophotometric/fluorescent properties. Although these methods allow the analysis of FAAH activity in multi-well plates, ideal for screening purposes, they use synthetic rather than the natural substrates. This is important considering the complexity of the cell and our motivation to investigate the metabolism in cellular settings that reflects the biological situation more.
Figure 2. URB597 inhibits AEA hydrolysis. The hydrolysis of [3H]-AEA during 15 min of incubation with and without 1 µM URB597 was measured in AT-1 cells pretreated in serum free medium supplemented with 0.001% BSA (vehicle), pH 7.4 (paper V). Blank are wells without cells. The graph represents mean ± 95% CI (N=6).

AEA uptake into intact cells (papers III, IV)

The assay, measuring AEA uptake is performed on intact cells by a protocol originally developed by Rakhashan et al. (2000) and modified by Sandberg and Fowler (2005). Cells are exposed to AEA tritium labelled in the arachidonic acid moiety ([Ara-3H]AEA), in KRH, pH 7.4 buffer supplemented with 0.1% fatty acid free BSA at 37°C for a specified time period. The uptake process is terminated by placing the reaction on ice, excess exogenous [Ara-3H]AEA is quickly washed off using KRH buffer supplemented with 1% BSA. Cell associated [Ara-3H]-AEA is released by lysing of the cells with 0.2 M NaOH. The tritium content of the lysate is quantified using liquid scintillation spectroscopy with quench correction. Inhibitors are added with a pre-incubation period of 10 min at 37°C before addition of [Ara-3H]AEA.

An important methodological issue with uptake (and hydrolysis) assays concerns the binding of substances to, and release from, the plastic in the wells. AEA, being a lipophilic molecule, binds very well to plastic. The plastic binding has been reported to vary greatly between 5-50% and depend upon varying factors such as presence of inhibitors, temperature and time (Fowler et al., 2004; Karlsson et al., 2004; Thors and Fowler, 2006). The addition of fatty acid free BSA (in the experiments of this thesis, a concentration of 0.1% is used) to the reaction buffer stabilizes AEA and prevents it from binding to the plastic in large amounts. In addition, BSA binds arachidonic acid, reducing the risk of product inhibition of FAAH (Omeir et al., 1995). However, on the other hand BSA also prevents AEA from entering the cell.
(Ligresti et al., 2004), and so the measured uptake reflects the concentration of free unbound AEA. To quantitate the plastic binding, wells without cells are included in each experiment.

Measurements of [Ara-\(^3\)H]AEA uptake do not take metabolic route into account. Thus, the tritium that is associated with the cell can consist of both non-degraded and degraded AEA as well as AEA degraded by other enzymes than FAAH. The inhibitory effect of URB597 on AEA uptake experiments is not complete (Figure 3). This may be due to an intracellular AEA binding reserve or a separate uptake mechanism independent of FAAH activity.

**Quantitative Polymerase Chain Reaction (qPCR) (papers III, IV, V)**

**RNA extraction**

Cells were washed once in cold PBS and lysed in cold lysis/binding buffer (100 µM Tris, 500 µM LiCl, 1% litium dodecyl sulphate, 5 mM dithiothreitol, pH 7.5). Lysates were stored at -80°C. Messenger RNA (mRNA) was isolated from thawed lysates using DYNABEADS® mRNA Direct kit according to the manual. Briefly the lysates are incubated with magnetic beads labelled with poly-T. A magnet is used to separate the beads from solute in a multistep wash procedure performed at room temperature. Finally, the samples are exposed to heat in sample buffer 10 mM Tris(hydroxymethyl)aminomethane (Tris), 1 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0) (80°C, 2 min) to dissociate the mRNA from the beads.

![Figure 3. URB597 inhibition of AEA uptake and hydrolysis in DU145 prostate cancer cells. Shown in the figure is mean + 95% CI (N=9). Data from paper IV.](image)
The extracted RNA concentration is estimated by absorbance (260 nm) using a Nanodrop Lite spectrophotometer. The purity of the samples was estimated by the 260/280 nm ratios and the cut-off value was defined as 1. A ratio >2 were considered good. Samples used in this thesis commonly had a value between 2.5-4.

**Complementary DNA (cDNA) conversion**

mRNA was transcribed to complementary cDNA using High-capacity cDNA reverse Transcription Kit (Applied Biosystems™). mRNA was mixed with master mix (deoxynucleotides (dNTP), random short primers, reversed transcriptase, RNase inhibitor, RT buffer and nuclease-free water). The thermal cycler conditions used for transcription was initially 25°C for 10 min followed by 37 °C for 120 min, terminating with 5 min at 85°C before a continuous 4 °C session was initiated.

The cDNA was diluted 1:10 in sample buffer (10 mM Tris, 1 mM EDTA, pH 8.0) to dilute interfering chemicals and save sample.

**Real time qPCR (RT qPCR)**

Real time quantitative amplification of mRNA was performed in an Eco Illumina system using SYBR green as the reporter dye. SYBR green binds double stranded genetic material and emits green fluorescence. Primers were designed in house and are intron skipping to avoid amplification of DNA. The primer sequences are listed in Table 1. Primer efficiencies were determined by cDNA dilution experiments. The efficiency of primer pairs targeting rat CB₁ and MAGL mRNA could not be robustly determined in cDNA from AT-1 cells.

The amplification scheme was as follows: activation phase: 95°C, amplification phase: (95°C: 10 sec, 60 °C: 30 sec)*45 cycles, 95°C: 15 sec. This is followed by a stepwise melt-session from 55°C to 95°C. Control samples lacking cDNA (no template control) were included as the final samples of every master mix preparation. Melt data acts as quality control to ensure amplification of single products and purity of control samples.
Table 1: Primer sequences targeting mRNA used in RT qPCR. Primers where designed in-house and are intron skipping to avoid amplification of contaminating DNA. Ef. = efficiency of the primer pair. *Efficiency could not be robustly determined. Primers used in paper IV and paper V.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Product</th>
<th>Forward primer (5’ to 3’)</th>
<th>Reverse primer (5’ to 3’)</th>
<th>Ef.</th>
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<td></td>
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<td>ABHD12</td>
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<tr>
<td>CPLA2G4A</td>
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<td>DAGLA</td>
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<td>CCCAAATGCGGAATCATCG</td>
<td>GGCTGAGAGGGCTTAGTATAG</td>
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<tr>
<td>DAGLB</td>
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<td><strong>Mouse</strong></td>
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<tr>
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</tbody>
</table>
| Gene          | Product     | Forward primer (5' to 3')               | Reverse primer (5' to 3')          | Ef.
|---------------|-------------|----------------------------------------|------------------------------------|------
| Abhd6        | ABHD6       | CATCTCGGCAGGAAGCTATAC                  | AGCAAGGTCTGGAGTGAAAG               | 90   |
| Abhd12       | ABHD12      | GACGTTCGCATCCCTGACACAC                 | CTGGTTGAGGCACTGGATCGTGTCC          | 93   |
| Cnr1         | CB₁         | GCTAGCTTCGGTTCGACATC                   | GGGAGAACCTGTATGAGGAAGAG            | *    |
| Cnr2         | CB₂         | GTGCTACCCACCTACCTAC                    | TGGAGATGACACGCGAGAG                | 103  |
| Dagla        | DAGLα       | TTTGAGCTGCTGGTCTCTG                    | CCAAGAGTACCTTGCCATTAG             | 99   |
| Daglb        | DAGLβ       | TTTGAGCTGCTGGTACAGA                   | CCAAGAGTACCTTGCCATTAG             | *    |
| Faah         | FAAH        | AAGGGCGGCGGTAGTGTAG                    | AATGATGCCAGCCAGAGGCC               | 107  |
| IL6          | IL-6        | GCCCTTCAGGAAGAGCTATG                   | GTGAAGTACGAGTTGAGACTG             | 90   |
| IL6R         | IL-6 receptor | CCACACAGTCTCTGTGTAAG                 | CCAGACACTTGCTTGGTCTCTG            | 92   |
| Mtll          | MAGL        | CGACTTTGAGAGAGCTCTTGTG                 | GATGAGTGATGCGGAGCTG               | *    |
| Naaa         | NAAA        | CGACTGAGAAGGGAACAGAC                  | TGGAGACTGACCTGCCATTAG             | 101  |
| Napepld      | NAPE-PLD    | ACTGTTACTGCCCTGCTTT                   | AATCCGACTGGACGGCGGAGG             | 94   |
| Psmc4        | 26S protease regulatory subunit 6B | CATCTGTCAGGAGGTGGAGATG             | GCTTTTCTGAAGTCCCTTG               | 96   |
| Ptgs1        | COX-1       | CGAGCCAGTACCAGGATC                    | AAGCGACCTGGAGAGCACCC             | 96   |
| Ptgs2        | COX-2       | TGAGCGGTACCACCTTCAA                   | TGCCAGTGATAGTGTTG                 | 91   |
| Rps12        | Ribosomal protein S12 | GACACCGAGCTCAACCTGTAAAG            | GCCATGTCCTTAACGCTAAACG             | 91   |
| Rpl19        | Ribosomal protein L19 | TACTGCAAACGGCTCCGAT             | AACAACCTACACCTGAGTGGC             | 93   |
The threshold for cycle quantification (Ct) data was set to 0.299-0.301 fluorescent units, cutting the amplification plots in the lower region of the exponential phase. The expression was calculated relative to the reference mRNA for ribosomal protein L19 (RPL19/Rpl19) (paper IV). RPL19 codes for one of two components in the 60S ribosome subunit. qPCR data is traditionally presented as $2^{-\Delta\Delta Ct}$, representing the difference between the target mRNA and the control mRNA, $(Ct_{\text{target}}-Ct_{\text{ref.}})_{\text{Target}}-(Ct_{\text{target}}-Ct_{\text{ref.}})_{\text{Control}}$. This way of interpreting the data gives a fold change of the target mRNA relative to the control mRNA, however it gives no information about the levels of the mRNA. In paper IV and V several target mRNAs were investigated. In order to represent both the level of mRNA and their changes, data is expressed as $\Delta Ct$ $(Ct_{\text{target}}-Ct_{\text{ref.}})$ on the left axis and $2^{-\Delta Ct}$ as percent of control on the right axis in the papers. One unit decrease in $\Delta Ct$ represents a doubling of the amount of mRNA. As an illustration, the two ways of presenting the data are shown in Figure 4.

![Figure 4. Two ways of displaying qPCR data. A) The traditional way of presenting qPCR data shows $2^{-\Delta\Delta Ct}$ on the y-axis and represents fold change. B) An alternative way of displaying qPCR data is to display $\Delta Ct$. A decrease of one unit represents a doubling of mRNA. Data is six separate samples isolated on the same experimental day (6 technical replicates).](image)

Although qPCR is an extremely useful technique, it is not without issues. An assumption is that the reference genes are expressed to the same extent regardless of the treatment undertaken. This is not always the case, not least in prostate cancer cell lines (Khimani et al., 2005). RPL19 mRNA levels in DU145 cells undertaken in paper IV, did not appear to vary to any great extent (see Figure 5A-B). However, when samples from several experiments were combined to give a large sample size, a significant difference was seen for LPS/IFNγ-treated RAW264.7 cells (Figure 5C).
This means that treatment-induced increases in mRNA levels will be slightly underestimated, and decreases over-estimated, and this should be borne in mind when interpreting small changes in mRNA levels of genes of interest. Due to uncertainties that the level of an mRNA is affected by a treatment it is often wiser to used multiple reference products when quantitating material. In paper V, three reference mRNAs were used (Figure 5D). The used reference mRNAs where 60S ribosomal protein L19 (\textit{Rpl19}), 40S ribosomal protein S12 (\textit{Rps12}) and proteasome subunit 26S ATPase4 (\textit{Psmc4}). The expression of target mRNAs was given relative to the average expression of all three reference mRNAs (termed r\textsubscript{all} in paper V). Additionally, the expression was calculated relative to all possible combinations of reference genes, and the statistics was determined in each case. The data are presented as r\textsubscript{all}, but the range of P values obtained using the other six combinations of reference genes give an idea as to the robustness of the P value for r\textsubscript{all}.

Two other issues that should be remembered are: I) that PCR amplifies genetic material; it is easy to make the assumption that the expression of a gene or the amount of mRNA for a specific protein correlates with the amount of protein in the cell. This is not the case, since posttranslational modifications can regulate the amount of mRNA that is translated to protein in the cell. II) RT qPCR is a very sensitive method able to detect very small differences in amount of starting material. It makes it powerful but also sensitive to contaminations and pipette errors. The preparation of material for qPCR involves many steps increasing the likelihood of errors.
Figure 5. Variations in levels of reference mRNAs used for normalization of target mRNAs in RT qPCR. Panels A and B; RPL19 was used as reference mRNA in DU145 cells treated with 20 ng/ml TNFα or vehicle (0.001% HSA). A) Variations in RPL19 during time-study (1-4 h, hourly sampling, 6 technical replicates) was analysed by a permutation ANOVA test a significant effect was seen over time but not for treatment (p=0.0046). B) RPL19 after 4 h of incubation with TNFα or vehicle (Data is pooled from several experiments to yield a high sample size). No significant difference determined by unpaired t-test with Welch’s correction for unequal variances. C) RAW264.7 cells were treated with 0.1 µg/ml LPS and 100 U/ml IFNγ in 1 µM NaHPO₄ pH 8.0 with 10⁻⁵ % BSA (final concentration) for 24 h. LPS and IFNγ increased the expression of Rpl19 (p=0.0021, unpaired t-test with Welch’s correction). D) AT-1 cells were treated with 0, 25 or 100 ng/ml IL-6 in PBS 0.001% BSA (final concentration) and modified KRH lactate buffer pH 6.6 or 7.4 for 3 h (6 separate experiments). pH 6.6 decreased the expression of Rps12 (p=0.021, permutation test). Data from paper IV and V.
Cyclooxygenase activity assay (papers I, II)

Figure 6 shows the reaction catalysed by the COX isoforms with arachidonic acid as substrate. For the purpose of measuring COX activity, a liquid-phase oxygen electrode chamber was utilised that measures changes in oxygen concentration. The method is essentially performed according to Meade et al. (1993) with minor adaptations. The oxygraph is calibrated to the ambient temperature and local air pressure.

The oxygen concentration of the substrate (10 µM, arachidonic acid or 2-AG) and substance (or substance and enzyme in time-dependency experiments) in reaction buffer at room temperature is established as the baseline. The reaction buffer (Tris-buffer) is supplemented with haematin (source of heme) and phenol for the sake of the enzyme activity (Smith and Lands, 1971). The addition of 200 units human recombinant COX-2 or ovine COX-1 (or 10 µM, arachidonic acid or 2-AG in case of time-dependency experiments) initiates the reaction, which was allowed to proceed for 5 min. The peroxidation reaction occurs without changes in oxygen tension. Thus, the assay detects oxygenative activity independent from the peroxidase activity. For classical NSAIDs, this is not an issue, but in theory additional effects of novel compounds upon the peroxidase activity would be overlooked.

**Figure 6. The reaction catalysed by COX-1 and -2 with arachidonic acid as substrate.** The enzymes consume oxygen in the first reaction step to give the unstable intermediate prostaglandin G₂ (PGG₂) that is thereafter metabolised by the peroxidative activity of the enzymes to give PGH₂. PGH₂ is converted by synthases to other prostaglandins. The oxygen electrode used for the experiments in this thesis is shown to the right.
The assay is calibrated to, and thus sensitive to the ambient temperature and the local air pressure and may fluctuate differently with different days. All experiments where performed in triplicates assayed on different days to avoid bias due to environmental variations.

Commercial COX-activity screening kits are available. The kits generally measures the peroxidase activity of the enzyme with colorimetric of fluorescence based endpoints. The commercial tests are expensive, often in need of extensive optimization, and do not allow comparison of inhibitory potencies towards different substrates. With commercial kits, one can often measure the COX- enzyme activity in cell lysates. Two such kits were tested in the project leading to paper IV, but neither worked satisfactorily.

**Western blot (papers III, IV)**

Before harvest, the cells were washed in cold PBS to remove dead cells and interfering components of the medium or treatment substance. Cells were harvested by scraping with a rubber policeman and lysed in 50 mM Tris, 150 mM NaCl, 1% Triton-x 100, pH 8.0 buffer with protease inhibitor and by sonication on ice. The protein content was determined in 96-well plates using Pierce™ BCA Protein Assay kit, a colorimetric method based on chelation between bicinchoninic acid (BCA) and copper. The assay is compatible with a number of chemicals, including 1% Triton X-100 used in the lysis buffer.

Samples were stored at -80°C until mixed in 5x Laemmli sample buffer, thereafter stored at -20°C. Protein were separated on stain-free precast gels (4-20%) and blotted on to mini polyvinylidene fluoride (PVDF) membranes (0.2 µm). Efficiency of protein separation and blotting was monitored using the Chemidoc MP system from BIO-RAD. Stain-free gels contain a fluorescence enhancing trihalo-compound that crosslinks to tryptophan when exposed to UV-light. Alternatives to precast stain-free gels are detection of housekeeping proteins such as actin and β-tubulin or Ponceau S staining of membranes. The precast stain-free gels require no extra chemicals or incubation/wash steps in contrary to antibodies for housekeeper proteins and ponceau staining (Rivero-Gutiérrez et al., 2014).
Membranes were blocked in TBST (500 mM NaCl, 0.1% Tween-20, 20 mM Tris, pH 7.4) with 5% fat free dry milk for 1 h. The membranes were exposed to the primary antibody overnight at 4 °C on a orbital shaker (COX-2; rabbit anti-mouse/rat raised in rabbit co-reactive with human, guinea, monkey, ovine and rabbit COX-2, cat#: 160106; Cayman Chemical Co., Ann Arbor, Michigan, USA). The horseradish peroxidase (HRP)-conjugated secondary antibody (polyclonal goat anti-rabbit immunoglobulin/HRP, Dako, Glostrup, Denmark) was applied for 1 h, room temperature. Membranes were washed in TBST, 6 x 5 min after each incubation and developed in electrochemiluminescence fluid and signals where detected using the BIO-RAD Chemidoc MP system, detection every 10 sec.

Western blot is not a sensitive method. The long protocol involves several steps where material is potentially lost. Western blot can be analysed subjectively by eye or digitized and quantified. The former is semi-quantitative, but simple when the question to be asked is: presence of protein yes/no; strong band/weak band. This was the case in the present thesis. The amount of protein to be loaded needs to be optimized for each protein source. Loading a small amount of protein may mask appearance of protein while loading high amounts will cause a larger loss of protein in transferring and thereby errors in assessment of amount of protein in samples. To detect COX-2 in DU145 cells up to 210 µg of whole cell lysate was loaded on the electrophoresis gel. This certainly raised the risk of loss of material during transferring.

**Thin layer chromatography (TLC) (paper IV)**

Lipids were extracted from 3.5-4 x 10^5 cells previously exposed to [Ara-^3H]AEA (tritium labeled in the arachidonic moiety) for 30 min (final concentration fetal bovine serum (FBS) 1.2% for DU145 and 10% for RAW264.7 cells). Lipids were extracted by scraping followed by extraction by the method of Bligh and Dyer (1959) using chloroform, methanol and water (3:2:1). Samples were vigorously shaken and water:oil phases were separated at 1000g x 5 min. Aliquots of the chloroform phase was applied to a 5 x 20 cm silica TLC plate (Silica Gel 150 Å, layer thickness 250 µm). Lipids were separated in 90% ethyl acetate, 10% methanol (Glass *et al.*, 2005) (total separation length was 16.25 cm). Strips (7.5 mm) of each lane were scraped using a scalpel. The scraped material was placed in scintillation vials and the tritium
signal was measured by liquid scintillation spectroscopy with quench correction. Untreated cell extracts were spiked with the standard compounds ([³H]-arachidonic acid, [Ara-³H]AEA, [³H]PGF₂α and [³H]bimatoprost (as a marker for PG-EAs) and added to silica plates as described above in order to establish the retention factor (Rᵢ) of each compound.

Scraping silica plates is time-consuming and the protocol included hazardous chemicals and materials. Ultra performance liquid chromatography tandem mass spectrometry (UPLC-MS/MS) is a more sensitive method to thin-layer chromatography but requires designated facilities and expertise. The method was used in paper III and IV in collaboration with Dr. Gouveia-Figueira, Department of Chemistry, Umeå University, Umeå, Sweden.

**Cell viability assays (paper V)**

**Lactate dehydrogenase (LDH) activity**

Lactate dehydrogenase (LDH) is an intracellular enzyme, present in more or less all cell types, that converts pyruvic acid to lactic acid as it converts NADH to NAD⁺. When the cell membrane is disrupted, LDH leaks to the extracellular medium. This is taken advantage of in the LDH activity assay that measures enzyme activity in medium samples where LDH activity induces a colorimetric change. In this thesis LDH activity was measured with the Cytotoxicity Detection Kit by Roche Diagnostics (Mannheim, Germany) according to the instructions. Included in each experiment were control samples of untreated cells, medium only and maximum dead cells (cells treated with 0.01% Triton-x 100 for 30 min at 37°C). The colorimetric change was quantified in a Spectrostar Nano-plate reader at 490 nm. LDH activity was calculated as % of maximum response (0.01% Triton-X 100 treated samples).

Extracellular LDH activity was measured in cells exposed to lactic acid. Control experiments indicated that the presence of lactic acid *per se* did not disturb the assay.
**Reduction of Thiazolyl Blue Tetrazolium Bromide (MTT)**

In metabolically active cells MTT is reduced to insoluble formazan precipitates (visible by microscope as purple precipitates inside the cell). These precipitates are solubilized and the collected accumulation is measured colourimetrically and correlated with cell viability.

The cells where incubated with 250 µg/ml MTT in fresh medium for 3 h (until dark precipitates had formed) at 37°C, 5% CO₂. The precipitate was solved overnight in sodium dodecyl sulfate (SDS) and HCl (added to the well (1:2), 5% SDS + 0.005M HCl). The plate was read in the Spectrostar Nano- plate reader at 570 nm. Included in each experiment were control samples containing untreated cells, medium only and maximum dead cells (cells treated with 0.01% Triton-x 100 for 30 min in 37°C). Data are presented as % of untreated cells.

Using several methods to detect cell viability increases the likelihood of a true result. Ideally, the MTT and LDH assays can be complemented with other methods, such as trypan blue exclusion by viable cells counted in automatic cell counters or Bürker chambers (paper III), and, for cells where a high number of apoptotic cells is anticipated, staining with propidium iodide (DNA stain, membrane impermeable) and annexin-V (phosphatidyl-serine, normally found only on the inner membrane), can distinguish viable, apoptotic and early/late apoptotic cells. Thus, each of the assays measures a specific activity within the cell, processes that may be changed for other reasons than cell death. Each of the methods also introduces new substances to the cell. These substances affect, and are handled by the cells in different ways. For example, the retention of MTT varies with the cell type tested, thus generalisations of absolute values between cell lines is not possible.

**Statistics**

The statistics involved in the analysis of the data in this thesis were undertaken using GraphPad Prism version 5 and 6 (GraphPad Software Inc., San Diego, CA. USA) or the statistical program R (R Core Team, 2017). The uncorrected alpha level was set to 0.05.
FAAH hydrolysis activity in paper I and II is presented as % of control with pI$_{50}$ and IC$_{50}$ values determined using GraphPad Prism non-linear log[inhibitor] versus response–variable slope (four parameters). Using this algorithm, the top value (no inhibition) is constrained to 100 and the “bottom” value (maximum attainable inhibition) was either constrained to 0 or allowed to float. The best fit was chosen by Akaike’s informative criteria (AIC). AIC is a mathematical method to compare the fit of a model to other models. AIC weighs the goodness of fit (sum of squares) to the number of parameters, punishing overfitting and rewarding good fit. AIC is only valid in comparison to other functions, where a lower AIC represent a more likely model. Prism reports the differences in AIC as the likelihood for each model to be correct in percent. The model with the best fit was used. Given that the model uses log[inhibitor], the primary outcome is pI$_{50}$, the negative log of the IC$_{50}$ value. Thus IC$_{50}$ is an extension of pI$_{50}$. IC$_{50}$ values and deviation measurements are back calculated from pI$_{50}$ when necessary.

The Hill slope also known as the slope factor (n$_{H}$) described in paper II relates to the slope of the dose-response curve and was originally used to describe cooperativity and binding of more than one ligand (Hill, 1913). The value can be positive (above zero) or negative (below zero) depending on the direction of the curve. A inhibitor dose-response curve has a negative n$_{H}$. Positive cooperativity, whereby the binding of one ligand facilitates the binding of other will result in an n$_{H}$ value >1. Likewise a value below 1 indicates negative cooperatively and that binding of one ligand hamper the binding of another. However, the n$_{H}$ values primarily represent the steepness of the inhibition curves and are not per se proof of cooperativity. In the present thesis, high n$_{H}$ values, generated through curve fitting in GraphPad Prism, are suggested to reflect binding of the inhibitor to BSA in the assay buffer mediating effects upon the free inhibitor concentration, rather than the presence of a cooperative interaction between inhibitor and enzyme (paper II).

K$_{i}$, the inhibitory constant, represents the concentration of inhibitor that reduces the enzyme reaction rate to half maximum velocity (V$_{max}$) under substrate-saturated situations. A second parameter, α relates to how inhibitor binding impacts upon the enzyme-substrate interaction, e.g how the inhibitor changes K$_{m}$. At α =1, the inhibition is non-competitive, whereas when α →∞, the inhibition is competitive. Competitive and mixed type inhibitions are the most common inhibitor modes. K$_{i}$
and α (mixed type inhibition) were calculated by GraphPad Prisms enzyme kinetics competitive and mixed model algorithms and from robust linear regression analysis of the intersects of the Dixon plot (paper II). When comparing fits, the best fit was chosen by AIC. Dixon plots, display \( \frac{1}{v} \ versus \) the concentration of inhibitor for varying substrate concentrations results in several lines that intersect with each other at \([I]=-K_i\) (except for uncompetitive inhibition, where the lines are parallel). Robust linear regression allows analysis of data where the residuals are unequal and non-normally distributed or when outliers cannot be removed.

COX oxygen consumption data was fitted to the plateau followed by one phase decay algorithm available in the GraphPad Prism program (paper I and II). The decay rate is calculated as the change in oxygen concentration between 10 and 30 sec after reaction is initiated (paper I and II).

The lipidomics data of paper III was analysed using a two-way robust Wilcoxon approach (rank based two-way ANOVA) or Kruskal-Wallis one-way ANOVA on ranks. The significance level is adjusted for multiple comparisons using the Bonferroni correction method. Thus with many comparisons in the same data set some comparisons will show a significant P value just by chance (type I error). The Bonferroni correction method generates a critical P value by dividing the significance level by the number of comparisons. The Kruskal-Wallis test was followed by Dunn´s multiple comparison post hoc test in GraphPad Prism version 6.0.

In paper IV (repeated measures) the lipidomics data had occasional missing values, precluding the use of repeated-measures ANOVAs. In this case a linear mixed multilevel model (function lme in the package linear and nonlinear mixed effects model (nlme) for R) allowing different sample sizes was used instead. The lipidomic data generates multiple P values and hence runs the risk of making type I errors. An alternative method to the Bonferroni method described above to control for false positives is to use critical P values calculated using a 5% false discovery rate by the Benjamini-Hochberg procedure (Benjamini and Hochberg, 1995). The method creates critical P values for each generated P value based on the position in a rank (low to high P value), the number of tests and the level of accepted false discoveries.
The test with the highest P value still below the critical P value and all tests with lower ranks are accepted as significant.

RT qPCR data was analysed using permutation tests (Fisher, 1935). At its simplest level (two samples) permutation tests randomly resample the dataset and calculate a distribution of possible means under the null-distribution. The fraction of permutations that are more extreme than the observed difference in mean gives the P value. The function (permTS; two sample permutation test) is found in the perm-package of R, together with lmp (linear model with permutation tests) followed by ANOVA of the lmPerm (permutation test for linear models) package. These tests were used for two independent (time versus mRNA expression) and two-way analysis (treatment, time and mRNA expression) respectively. A Monte-Carlo approach, resampling randomly 10 000 times, was used when the total number of possible iterations was too high to be feasible. Permutation tests are useful since they make fewer assumptions about the dataset than standard parametric tests, but they are still sensitive to heteroscedasticity (unequal variances) in unbalanced datasets and to the presence of outliers. Simple comparison between two groups was analysed using parametric-unpaired two-sample t-test (GraphPad Prism version 6.0).

Another non-parametric permutation test is the bootstrap method used for uptake and hydrolysis experiments in paper IV. When using bootstrap methodology, the samples are resampled with replacement (each sample can occur multiple times in the bootstrapped sample) a fixed number of times. For each bootstrap-sample a t-value is calculated. The P values are calculated as two times the number of resampled tests that had a t-value more extreme than the original t-value under the null-hypothesis divided by number of resamples (in this thesis 10 000 in all permutation tests). In paper V, the hydrolysis data was analysed by a two-way ANOVA corrected for multiple comparisons using Tukey’s test in GraphPad Prism, version 6.0 d.

Cytotoxicity data (LDH assay) was analysed with a permutation ANOVA on unmatched data. Cell viability (MTT reduction) is analysed by parametric one sample t-test, compared to control, defined as 100%.
Results

Dual FAAH/COX-2 substrate-selective inhibitors

Metabolites of ibuprofen and flurbiprofen (paper I)

The most abundant metabolites of flurbiprofen; 4´hydroxyflurbiprofen and ibuprofen; carboxy- and 2´hydroxyibuprofen and the minor metabolites 1´-, and 3´-hydroxyibuprofen (Figure 1) all showed pH dependent inhibition of FAAH-mediated hydrolysis of AEA.

All metabolites were more potent at pH 6.0 than at 7.3, consistent with ibuprofen and flurbiprofen mediated FAAH inhibition (Holt et al., 2001; Fowler et al., 2003). None of the compounds were more or equally potent as the parent compound (Figure 2). Hydroxylation of flurbiprofen in position 4 decreased the FAAH inhibitory potency 3-fold (IC₅₀: 84 versus 28 µM for the metabolite and parent
compound, respectively) at pH 6.0. Ibuprofen metabolites showed IC$_{50}$ values in the range of 200-410 µM at pH 6.0 compared to 70 µM for ibuprofen at the same pH.

Complete inhibition of COX-1 was produced by 30 µM flurbiprofen and 300 µM ibuprofen. The ibuprofen metabolites 1’hydroxy-, 2’hydroxy-, 3’hydroxy- and carboxy-ibuprofen did not inhibit COX-1 to any great extent at any concentration. 3’ Hydroxyibuprofen retained the ability of ibuprofen to inhibit COX-2 oxygenation of 2-AG, although the inhibition was not complete at the highest concentration tested (300 µM). 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 70%, confirming the substrate selective inhibition of COX-2 by this compound (Duggan et al., 2011; Cipriano et al., 2013). 4’-Hydroxyflurbiprofen retain the substrate specific inhibition of COX-2 thus inhibiting COX-2 cyclooxygenation of 2-AG completely at 300 µM but failed to inhibit COX-2 catalysed oxygenation of arachidonic acid. However, COX-1 was completely inhibited at 1 mM (Table 1). 1’hydroxy-, 2’hydroxy and carboxy-ibuprofen lacked inhibitory effects upon the COX-2 isoenzyme regardless of the substrate used.
**Table 1. Inhibition of COX-1 and 2 utilisation of arachidonic acid (AA) and 2-arachidonylglycerol (2-AG) by the metabolites of ibuprofen and flurbiprofen.** The % inhibition (change in O₂ concentration) between 10 and 30 sec after addition of the enzyme produced by 100 µM compound (unless otherwise shown) are given (means ± 95% CI, N=3)

<table>
<thead>
<tr>
<th>Inhibition % @ 100 µM ± 95% CI</th>
<th>COX-1</th>
<th>COX-2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA</td>
<td>2-AG</td>
</tr>
<tr>
<td>ibuprofen</td>
<td>74 ± 10</td>
<td>74 ± 4 @30 µM</td>
</tr>
<tr>
<td>1´OH ibu</td>
<td>-12 ± 41</td>
<td>-3 ± 42</td>
</tr>
<tr>
<td>2´OH ibu</td>
<td>-2 ± 34</td>
<td>-25 ± 47</td>
</tr>
<tr>
<td>3´OH ibu</td>
<td>19 ± 31</td>
<td>-35 ± 54</td>
</tr>
<tr>
<td>carboxy</td>
<td>-10 ± 44</td>
<td>-5 ± 38</td>
</tr>
<tr>
<td>flurbiprofen</td>
<td>103 ± 13</td>
<td>70 ± 29 @3 µM</td>
</tr>
<tr>
<td>4´OH flurbi</td>
<td>3 ± 60</td>
<td>76 ± 8</td>
</tr>
</tbody>
</table>

**Figure 3. Chemical structure of ibufenac-AM1, and flu-AM1 and ibu-AM5 derivatives of flurbiprofen and ibuprofen respectively.** * Marks the position of the chiral center. Ibufenac-AM1 lacks the chiral center of Ibu-AM5. Structures were created in open-source molview v2.4.
FAAH inhibition by the enantiomers of flu-AM1 and ibu-AM5 (paper II, III)

The structures of ibu-AM5, flu-AM1 and ibufenac-AM1 are shown in Figure 3.

Both the (R)- and (S)- enantiomer of flu-AM1 inhibit FAAH at low micromolar concentrations in rat brain homogenates (IC$_{50}$=0.74 and 0.99 µM, respectively) but were 10 times less potent in mouse brain homogenates (IC$_{50}$=8.8 and 11 µM, respectively). The (S)-enantiomer of ibu-AM5 was 10 times as potent as the (R)-enantiomer and displays the species difference seen for flu-AM1 (IC$_{50}$= 0.59 versus 5.7 µM (rat brain) and 7 versus 53 µM (mouse brain)). The methyl group at the chiral center of ibu-AM5 is important for the inhibitory activity. Thus, ibufenac-AM1 (IC$_{50}$ 68 µM) lacking the methyl group was considerable less potent than the ibu-AM5 enantiomers and the racemat (Figure 4). Ibu-AM5 racemate showed, as expected, an intermediate potency (IC$_{50}$ 1.2 µM).

Inhibition dose-response curves from experiments with varied substrate and inhibitor concentrations was fitted to models of mixed and competitive inhibition modes and compared by AIC. Both substances fitted a mixed mode of inhibition better. (S)-ibu-AM5 inhibited rat brain AEA hydrolysis with $K_i$ and $\alpha$ values of 0.80±0.21 µM and 3.2±1.5, respectively. $\alpha$ correspond to the change in $K_m$ that is introduced by the inhibitor, in practice $\alpha$ describes the change in enzyme affinity for

![Figure 4. Inhibition of FAAH by ibufenac-AM1 and the enantiomers of flu-AM1 and ibu-AM5. Inhibition of 0.5 µM [3H]AEA in FAAH preparation form rat brain homogenates. Data is presented as % of hydrolysis by uninhibited enzyme with the upper and lower range (N=3).](image-url)
the substrate that is induced by the inhibitor. The corresponding values for the (R)-
enantiomer were 10±2.5 µM and 3.1±1.1, respectively. The Dixon-plot displays 1/v
versus [Inhibitor]. $K_i$, found at the x-axis ([inhibitor]) where the lines intersect
generated $K_i$ values of 0.89 and 9.8 µM for (S)- and (R)-ibu-AM5, respectively
(Eisenthal and Cornish-Bowden, 1974).

In rat brain homogenates, the inhibition by (R)-flu-AM1 is slightly time-dependent
but fully reversible (Figure 5). Similar to the enantiomers of ibu-AM5, (R)- and (S)-flu-AM1 inhibition of AEA hydrolysis fits a model for linear mixed inhibition better
than a competitive interaction model. $K_i$ and $\alpha$ values for (R)- and (S)-flu-AM1 were
0.63±0.17 µM, 3.2±1.3 and 0.79±0.28 µM, 5.6±3.6, respectively. The Dixon-plot
generated $K_i$ values of 0.28 and 0.86 µM for (R)- and (S)-flu-AM1, respectively
(paper II). In mouse brain homogenates (R)-flu-AM1 behaved as a competitive
inhibitor of FAAH with a $K_i$ value of 20±8 µM, confirmed by Dixon plot ($K_i$ value
=19 µM) (paper III).

**Cooperativity in (R)-flu-AM1 and carprofen binding to FAAH (paper II)**

The Hill slope ($n_H$) can be used to estimate the cooperativity of the enzyme-inhibitor
interaction. The $n_H$ values for both enantiomers of ibu-AM5 and flu-AM1 were
essentially 1 for rat brain FAAH, i.e. no cooperativity. Carprofen on the other hand
inhibited rat brain FAAH with a $IC_{50}$ value 28 µM and a $n_H$ value of 1.66±0.13,
indicating positive cooperativity ($n_H$ was derived from data points for carprofen
versus FAAH dual inhibitor data, [second inhibitor] =0). Further analysis
conducted primarily by co-author Fowler demonstrated that the high $n_H$ seen with
carprofen could be a result of its binding to serum albumin affecting the free
carprofen concentration, rather than a true positive cooperative interaction between
inhibitor and enzyme.
Figure 5. Characterisation of FAAH inhibitory mode of action by (R)-flu-AM1. (R)-flu-AM1 and URB597 in increasing doses were incubated with rat brain homogenates and [3H]AEA for 0, 15, 30, 45 and 60 min. For each concentration of inhibitor, the time-dependent slope was calculated by linear regression curve fitting. The slopes represent the change in hydrolysis rate and are presented as mean with 95% CI, N=3. Slope=0 indicates no time-dependency. B) Reversibility of FAAH inhibition by (R)-flu-AM1. Rat brain homogenates (20-fold standard concentration) were pre-incubated with high concentration (0, 2, 4 or 6 µM) (R)-flu-AM1 for 60 min. Aliquots were then diluted 20-fold to low concentration, new concentration: (0.1, 0.2 and 0.3 µM) and assayed for FAAH activity according to standard protocol. Included in the hydrolysis experiment was also (R)-flu-AM1 (0.1, 0.2, 0.3, 2, 4 and 6 µM) added to vehicle preincubated aliquots. 0.1, 0.2, 0.3 µM samples represented free concentrations after the 20-fold dilution of 2, 4 and 6 µM final concentrations, in case of reversible binding mode. The panel shows the data as % of corresponding control, error bars indicate the range. Llines are fitted by linear regression and are included to ease the interpretation (N=3). For a fully reversible compound, the diluted samples should behave as the low concentration samples.
Inhibitor-inhibitor competition experiments (paper II)

Dual-inhibitor experiments can be used to determine whether or not the bindings of the inhibitors to the target enzyme are mutually exclusive (Segel, 1975). In the experiments in paper II, the concentrations of carprofen and (R)-flu-AM1 or (S)-ibu-AM5 were varied and the data were visualized in Dixon plots. If the inhibitors act mutually exclusively when binding FAAH, the Dixon plot reveal a series of parallel lines with equal slope. Two compounds interacting in a cooperative manner result in lines forming a “V”-shape towards the y-axis (Segel, 1975). The Dixon plot suggest that the compounds act upon FAAH in a mutually exclusive manner meaning the binding of one molecule blocks the binding of another molecule (Figure 6).

Figure 6. (R)-flu-AM1 and carprofen inhibition of FAAH in a multiple-inhibitor experimental setup. (R)-flu-AM1 and carprofen were combined in different concentrations and the FAAH hydrolysis of 0.5 mM [3H]AEA was measured in rat brain homogenates. The results are displayed as mean with 95% CI, N=3. A) Dixon plot displaying one over the rate of hydrolysis versus the concentration of (R)-flu-AM1 for each concentration of carprofen. B) replot of the slopes obtained in A.
**COX inhibition by (R)- and (S)-flu-AM1 (paper III)**

Both (R)- and (S)-flu-AM1 inhibited 2-AG oxygenation by human recombinant COX-2 (1 \textit{versus} 0.7 µM) and arachidonic acid oxygenation by COX-2 and ovine COX-1. However, the (S)-enantiomer was roughly twice as potent as the (R)-enantiomer when arachidonic acid was the substrate (10 \textit{versus} 20 µM for COX-2 and 3 \textit{versus} 6 µM for COX-1), but both enantiomers showed substrate-selective inhibition of COX-2. The oxygenation of 2-AG was inhibited at concentrations an order of magnitude lower than required for inhibition of arachidonic acid by COX-2 (Figure 7). The inhibition of COX by flurbiprofen is time-dependent and preincubation of COX-1 and -2 with (R)- and (S)-flu-AM1 increased the inhibition of

\[ \text{Figure 7. COX inhibition of the (R) and (S) enantiomer of flu-AM1. Activity is measured as change in oxygen tension over 2 min following addition of the concentrations (µM) shown in the figures. COX-1 is of ovine origin and utilizes arachidonic acid as substrate (A). COX-2 is human recombinant and oxygenates 2-AG (B) and arachidonic acid (C) (N=3). Initial reaction rates (lag phase + 1 sec) were used to derive IC}_{50} \text{ values.} \]
arachidonic acid and 2-AG (for COX-2) cyclooxygenation, thus suggesting a time-dependent mode of action (paper III) (Rome and Lands, 1975). The found FAAH:COX-2 inhibitor profile of \((R)\)-flu-AM1 is favorable for increasing eCB tonus while leaving the prostaglandins unaffected. Next the contribution of COX-2 and the effect of \((R)\)-flu-AM1 upon AEA levels in intact cells were tested.

**COX-2 and its contribution to AEA degradation in intact cells**

*COX-2 in mouse leukemic RAW264.7 macrophage cells (papers III, IV)*

Using a cell line where the expression of COX-2 can be induced allows elucidation of the COX-2 contribution to AEA turnover in settings of high and low expression. In paper III and IV the expression of COX-2 was upregulated in mouse macrophage cell line RAW264.7 cells by treatment with 0.1 µg/ml LPS and 100 U/ml of IFNγ for 24 h. The treatment produced a large increase both in COX-2 mRNA and in protein content visualized by Western blot (paper III, IV). The treatment also increased the FAAH mRNA expression and decreased NAAA expression. A slight reduction in the total protein recovered from the wells was also found.

UPLC-MS/MS experiments undertaken by co-author Gouveia-Figueira showed that the increase in COX-2 mRNA was accompanied by increased levels of prostaglandin D₂ (PGD₂) and PGE₂ in cell lysates. LPS and IFNγ treated cell also contained higher levels of 11-HETE, 15-HETE, a small increase in AEA and a small decrease in 2-AG. Flurbiprofen (10 µM) and \((R)\)-flu-AM1 (10 µM) reduced the levels of PGD₂, PGE₂ and 11-HETE in both stimulated and unstimulated RAW264.7 cells but left 2-AG and AEA levels unaffected. No lipids could be detected in the medium during these experiments. Neither did stimulation with the calcium ionophore ionomycin (5 µM) that stimulates AEA synthesis and release (Di Marzo et al., 1994) generate any detectable lipids in the medium of vehicle treated cells, although low levels of PEA, SEA, OEA, 13-HODE and 9(S)-HODE was detected in the medium of LPS and IFNγ treated cells. Neither flurbiprofen nor \((R)\)-flu-AM1 had any effect upon AEA and 2-AG levels.

To see whether COX-2 affects the catabolic balance of AEA, LPS and INFγ treated RAW264.7 cells were exposed to \[^3^H\]AEA (100 nM for 60 min) in the absence or
presence of flurbiprofen (10 µM) and/or URB597 (1 µM) or (R)-flu-AM1 (10 µM). URB597 with and without flurbiprofen completely blocked AEA hydrolysis. Flurbiprofen alone and (R)-flu-AM1 did not inhibit AEA hydrolysis, thus confirming the results seen in UPLC-MS/MS experiments (paper III). Surprisingly, URB597 only produced a minor effect upon the levels of AEA and related N-acylethanolamines in the cells, measured by UPLC-MS/MS. In addition, ionomycin only produced a small increase in AEA, PEA, and SEA levels in the cell when combined with URB597 but not (R)-flu-AM1. Thus, at a concentration of URB597 causing complete inhibition of the hydrolysis of exogenous AEA, endogenous levels were only marginally affected. Uptake experiments in paper IV likewise showed that flurbiprofen did not affect AEA uptake and the effect of URB597 was modest.

To investigate the fate of AEA ([Ara-3H]AEA) after 30 min incubation with intact RAW264.7 cells, lipid extracts where separated by TLC with 90% ethyl acetate and 10% methanol (Glass et al., 2005). Tritium labelled bimatoprost (17-phenyl trinor prostaglandin F$_{2\alpha}$ ethyl amide), AEA, arachidonic acid and PGF$_{2\alpha}$ was used as references. The recovery was low in preliminary experiments of vehicle-treated RAW264.7 cells. Higher recovery and elution concomitant with bimatoprost was seen in LPS and IFNγ-treated cells consistent with a higher expression of COX-2 indicating that AEA is indeed bioactivated by COX-2 in these cells.

**COX-2 in human DU145 prostate carcinoma cells (paper IV)**

The effects of COX-2 upon AEA metabolism may vary with the tissue and organ. Next, we examined whether COX-2 is a significant contributor to AEA breakdown in human prostate cancers cells given the association of COX-2 and prostate cancer. Consistent with previous published data (Subbarayan et al., 2001), 20 ng/ml TNFα increased the mRNA levels of COX-2 (PTGS2). The upregulation was visible after 1 h and maintained for 4 h, the longest time point investigated. Compared to the Ptgs2 levels in RAW264.7 cells, the expression and the increase of PTGS2 was low and COX-2 protein levels were below the limit of detection in Western blots. The mRNA levels of FAAH and NAAA remained unchanged throughout the treatment session while the levels of NAPE-PLD decreases.
TNFα-treatment of DU145 did not affect the protein content in the wells and both hydrolysis and uptake was significantly reduced by URB597 regardless of treatment. Flurbiprofen reduced the hydrolysis of AEA by ~10%, but did not affect the uptake.

The recovery in TLC separation, and detection of bimatoprost (17-phenyl trinor prostaglandin F₂α ethyl amide), AEA, arachidonic acid and PGF₂α in lipid-extracts after a 30 min incubation with [Ara-³H]AEA was low in vehicle- and TNFα-treated DU145. UPLC-MS/MS experiments confirmed the lack of prostaglandin and prostaglandin ethanolamide (PG-EA) detection in treated and untreated DU145 cells. Further, AEA levels were very low and the NAE (AEA, PEA, OEA and LEA) levels in the cell lysates did not differ between TNFα- and vehicle-treated DU145 cells. However exogenously added AEA (100 nM added at the 4 h time point) was rapidly taken up by the DU145 cells and production of PGE₂ and PGD₂, thromboxane B₂, PGE₂-EA and 15-HETE could be detected in both the medium and the cell extracts of treated and untreated cells (samples collected over the following 2 h). PGE₂-EA was also cleared from the medium, albeit more slowly than AEA.

**Hydrolysis of AEA by rat AT-1 prostate carcinoma cells (paper V)**

It has previously been shown that rat R3327 Dunning AT-1 prostate carcinoma cells can accumulate and hydrolyse AEA (Thors *et al.*, 2007), but those experiments were undertaken using standard culturing conditions, rather than conditions relevant to solid tumours in vivo. In consequence, the turnover of AEA was investigated in AT-1 cells exposed to IL-6 (0, 25 and 100 ng/ml) and lactic acid (pH 6.6 or pH 7.4) in low serum conditions for 3 h. At the mRNA level, the expression of Naaa was high compared to Faah (Ct 7 versus 12.9 respectively). Neither treatment affected the mRNA expression of the synthetic enzyme Nape-pld, nor Faah or Naaa. In functional assays, hydrolysis of AEA by intact cells was brought about by FAAH rather than NAAA, and was not affected by the lactic acid treatment.

**2-AG and its metabolizing enzymes (papers IV, V)**

This thesis focuses upon AEA metabolism. However, enzymes related to 2-AG and 2-AG levels were also measured. 2-AG related enzymes were included in RT qPCR performed in human and rat prostate cancer DU145 and AT-1 cells. TNFα treatment
of DU145 reduced the mRNA levels of the 2-AG synthetic enzymes DAGLa and DAGLb ≥ 2 h of treatment. There was no significant effect upon the mRNA levels of the hydrolytic enzyme MAGL, ABHD6 or -12. However, despite being unaffected by TNFα, the mRNA levels of MGLL and ABHD12 were considerably higher than for FAAH, NAAA and PTGS2. Possibly reflecting a higher turnover of 2-AG and in the case of DAGLA/B reduction a decreased production of the same.

In contrary, in AT-1 cells, 3 h of treatment with lactic acid (pH 6.6) significantly increased the expression of Dagla (Figure 8). This could suggest that the 2-AG system, rather the AEA system is sensitive to the tumour environment.

![Graph showing mRNA expression of 2-AG related target genes](image-url)

**Figure 8. mRNA expression of 2-AG related target genes in rat prostate cancer cells.** Rat prostate cancer cells were treated with pH 6.6 induced by lactic acid and IL-6 in a serum free environment for 3 h. The mRNA expression of eCB related genes were measured by RT qPCR. Shown in the figure are the levels of 2-AG synthesising (Dagla and Daglb) and 2-AG degrading (Mgll, Abhd6 and Abhd12) enzymes relative to the levels in control cells (pH 7.4, 0 ng/ml). The result is displayed as mean and minimum to maximum values (data from six separate experiments). The efficiency of Mgll primers could not be robustly determined.

**Toxicity of enzyme inhibitors (paper V)**

To investigate functional effects of the changes in mRNA levels upon cell viability, AT-1 cells were exposed to lactic acid (pH 6.6) in combination with inhibitors of eCB
related enzymes for 24 h. The cell viability was investigated by quantification of extracellular lactate dehydrogenase (LDH) activity and reduction of thiazolyl blue tetrazolium bromide (MTT). The lipase inhibitor orlistat (20 µM) doubled extracellular LDH activity (P=0.0036) indicative of a higher frequency of cell death (Figure 9B). However, the change was not significant when applying false discovery corrections (critical P value =0.0033). The MTT assay also indicate a higher metabolic activity in orlistat treated cells (Figure 9A).

![Figure 9](image_url)

**Figure 9. Quantification of LDH release and MTT reduction for assessment of cell viability.** AT-1 cells were exposed to pH 7.4 or pH 6.6 induced by lactic acid in combination with inhibitors of eCB related enzymes for 24 h. U= URB597, F= flurbiprofen, (R)=(R)-flu-AM1, O= orlistat and W=WWL70, concentrations are µM. Graphs show mean with 95% CI, N=6. The α level is corrected for multiple comparisions using the Benjamini-Hochberg procedure. A) MTT reduction as a correlator of metabolic activity, expressed as procent of vehicle treated cells. Data is analysed by one sample t-test compared to 100%. Critical P-value = 0.0083, *P<0.05, **P<0.0083. B) LDH release as a correlator of cell death, expressed as percent of maximum LDH release induced by 0.01% Triton-X treatment for 30 min prior to analysis. Data is analysed by permutation ANOVA, critical P-value 0.0033.
Discussion

Even though studies show that the levels of AEA are increased by FAAH inhibition or knockout, the elevated levels of AEA may be suboptimal due to an increased degradation via other catabolic pathways. One such catabolic pathway, not least due to its induction in inflammatory conditions, is the bioactivation pathway by which AEA is converted to prostamides by COX-2. In the present thesis, the roles of FAAH and COX-2 in the catabolism of AEA have been explored pharmacologically both in normal conditions and following inflammatory stimuli.

In addition, ibuprofen and flurbiprofen metabolites and derivatives are examined and characterized regarding their FAAH and COX inhibitory properties in the search for a new improved analgesic. Thus, FAAH inhibition is linked to therapeutic effects such as analgesia and anti-inflammatory effects that is to some extent independent of CB receptor activity (Lichtman et al., 2004a). Thus, the effects may be attributed by other targets of AEA, such as TRPV1. In addition, FAAH hydrolyses NAEs, thus the effect could partly be mediated by other NAEs such as the anti-inflammatory compound PEA.

Endocannabinoid targeting dual-action inhibitors are also investigated by others; Adamson Barnes et al., (2016) report that FAAH/MAGL inhibition produce in vivo effects in a neuropathic pain model; dual FAAH/TRPV1 inhibitors mediate analgesic effects in osteoarthritis models (Malek et al. 2015). No inhibitors of NAAA/FAAH have yet emerged.

Dual FAAH-COX-2 substrate selective inhibitors

Paper I explore the FAAH and COX inhibitory profiles of the major metabolites of ibuprofen and flurbiprofen at different pH, the rationale being that pharmaceuticals may be degraded to metabolites with a changed activity profile for target molecules or other molecules (for example morphine, Wittwer and Kern, 2006). An increased pattern of FAAH inhibitory potency for the profen metabolites would suggest that an eCB component following administration of the parent compounds might be expected in vivo.
Paper I show that the major metabolites of ibuprofen and flurbiprofen inhibit FAAH and COX-2 and retained the pH dependency of FAAH inhibition displayed by parent compound although at lower potency. All metabolites except 3´hydroxyibuprofen and 4´hydroxyflurbiprofen lost the substrate selective inhibition of COX-2 favoring 2-AG. However, the effect seen by 3´hydroxyibuprofen was not visible in the initial reaction velocity. This may be linked to a common interaction for both the substance that induce a steric hinder for the otherwise flexible Leu\(^{552}\), permitting multiple substrates. 3´hydroxyibuprofen and 4´hydroxyflurbiprofen both have the hydroxyl- group extending outwards, making the molecule longer and more hydrophilic. The hydroxylation of the other metabolites may add to a bulkiness that disturbs the interaction within the channel. However, the potencies are low and none of the metabolites are likely to mediate any therapeutic effects in vivo.

In paper II and III, the enantiomers of flu-AM1 and ibu-AM5 where tested for their FAAH/COX inhibitory effects. In both compounds, the carboxyl-group is changed to an (3-methyl-pyridine-2-yl)-acetamide group. The structural change greatly increases the potency towards FAAH. The two enantiomers of flu-AM1 had rather similar potencies towards FAAH, whereas the (S)-enantiomer of ibu-AM5 was more potent than the corresponding (R)-enantiomer. This is in the opposite direction to that seen with ibuprofen itself (Holt et al., 2001). Modeling studies conducted in paper II suggested that the substances bind a site overlapping with the binding site for carprofen located between the two substrate-access/binding channels (Bertolacci et al., 2013). This was supported by dual-inhibitor experiments where the binding of (R)-flu-AM1 reduce the binding of carprofen and vice versa. The two ibu-AM5 enantiomers fit very differently into the active site of FAAH. Thus, the binding modes of the enantiomeric forms of flu-AM1 and ibu-AM5 to the rat FAAH reflects the difference in potency observed. All conformations tended to favour a binding mode where the amide points towards the active site with H-bonds to Thr\(^{488}\) and Gly\(^{485}\). This was supported by decreased potency towards mutant FAAH\(^{T488A}\) of (R)-flu-AM1. The (S)-conformation of both flu-AM1 and ibu-AM5 bind deeper within the FAAH active site channel. Both flu-AM1 enantiomers make similar interactions with FAAH and this is functionally represented by similar FAAH inhibitory potentials. (R)-ibu-AM5 is dramatically less potent than the other substances. This may simply be due to a reduced number of hydrogen bonds. Interestingly, ibufenac-AM1, lacking the chiral center of ibu-AM5 was less potent than either ibu-AM5
enantiomer. Thus, the presence of the methyl-group (in both ibu-AM5 and flu-AM1) rather than its configuration seems important. In addition, no specific interaction was made with the methyl-group.

In conclusion, the results showed that (R)-flu-AM1 displayed a promising profile as an eCB selective COX-2 inhibitor. This substance was further evaluated in intact cells.

The role of COX-2 in the degradation of AEA

In RAW264.7 cells treated with LPS, Nape-ipld is down regulated and Faah induced or unchanged (Liu et al., 2003; Murakami et al., 2007) in contrast to a large induction of Ptgs2 (COX-2) when combined with INFγ (Von Knethen et al., 1999). LPS is also reported to increase release of arachidonic acid and subsequently increase AEA synthesis in RAW264.7 cells presumable via phospho-AEA (Pestonjamasp and Burstein, 1998; Liu et al., 2004). In our hands, COX-2 was as expected greatly upregulated by the treatment with LPS and IFNγ, and a peak co-eluting with the prostamide bimatoprost was detected in TLC separation experiments for RAW264.7 cells incubated with AEA, indicating functionality in the enzyme (paper IV). (R)-flu-AM1 failed to affect eCB levels but efficiently blocked the production of prostaglandins. A similar result was seen with flurbiprofen, even when combined with URB597 (paper III). (R)-profens has previously shown to selectively inhibit eCB degradation via COX-2 in mouse dorsal root ganglion cells (Duggan et al., 2011). The discrepancy between our results implies that COX-2 is not a major contributor to eCB degradation in RAW264 cells. The same is true for the uptake of AEA, where a COX inhibitor-sensitive component of the uptake was not seen (in contrast to the FAAH inhibitor-sensitive component), even following COX-2 induction.

There is a discrepancy between the results of (R)-flu-AM1 in the isolated enzyme assays (with human recombinant COX-2) and the intact mouse RAW264.7. The low potency of the compound towards COX-2 in the enzyme assay when arachidonic acid is used as substrate and yet there is a pronounced inhibition of prostaglandin production in the intact LPS + IFNγ-treated cells. An issue not to be overlooked when considering differences of this type is the difficulty of extrapolating results
between isolated enzyme assays and intact cells. In experiments using intact cells, intracellular macromolecular crowding needs to be considered. Crowding of proteins, enzymes, DNA, RNA cellular building blocks and compartments reduces the exposure to solvents. The change in viscosity affects the dynamics of interactions in several ways. Thus, a ligand in near distance of its target protein is more likely to interact with said target due to proximity and time issues. Thus, the kinetics and dynamics observed in an isolated enzyme assay, where the viscosity and presence of other components is limited may not represent the events in an intact cell. It can be speculated that in the vicinity of COX-2 in the intact cell, the concentration of arachidonic acid is lower, and (R)-flu-AM1 higher, than in the cell-free systems, thus producing inhibition of prostaglandin production in the former but not the latter.

COX-2 mRNA levels were upregulated in DU145 cells treated with TNFα or vehicle (paper IV). However, no COX-2 enzyme could be detected in Western blots. In LPS-treated but not in vehicle treated RAW264.7 COX-2 was readily detected consistent with the observed upregulation of mRNA. Even though the level of COX-2 was undetectable, AEA was readily taken up and degraded and prostamides and prostgaldnins could be detected after addition of exogenous AEA (100nM). AEA may have contributed to prostaglandin production via arachidonic acid release through hydrolysis of FAAH.

The cells where extracted using the same protocol and were analysed simultaneously with the same antibody dilutions. Thus, the absence of detection in DU145 cells is most likely not an experimental error. The level of mRNA was lower in DU145 cells than in RAW264.7 cells, thus the lack of detection may be due to low, by Western blot undetectable amounts of COX-2. Another possibility is that post-translational modifications degrade or inactivate the mRNA or degrade the enzyme when produced (reviewed by Maier et al., 2009). The discrepancy between the Western blot and UPLC-MS/MS results thus most likely reflects the different sensitivities in the two methods.

Effects of inflammatory factors upon eCB related enzymes

Given the link between cancer and inflammation, tumour cells were treated with inflammatory factors both to elucidate COX-2 involvement in AEA degradation and
to explore extracellular factors that may be responsible for the changes in the eCB system that is observed in cancer.

Human prostate DU145 and mouse macrophage RAW264.7 cells were exposed to TNFα and LPS+IFNγ, respectively, to upregulate COX-2 (Subbarayan et al., 2001; Von Knethen et al., 1999). The induction of COX-2 was stronger in RAW264.7 cells, likely reflecting its role in the immune system. IL-6 and lactic acid exposure of AT-1 cells did not affect the COX-2 expression. Although, the COX-2 mRNA expression was homogenous with a large spread in both treated and untreated samples (paper V).

All three cell lines (rat AT-1, mouse RAW264.7 and human DU145 express higher amounts of NAAA than FAAH mRNA. In DU145 and AT-1 cells the expression was unchanged by the treatment with inflammatory components. However, in RAW264.7 cells the expression of FAAH increased and the expression of NAAA decreased upon exposure to LPS and IFNγ, shifting the balance. Despite the higher expression of NAAA than FAAH in AT-1 cells, the latter was primarily responsible for the hydrolysis of AEA consistent with the small preference of NAAA for AEA.

AT-1 rat prostate cancer cells express mRNA for IL-6 and its receptor in accordance with findings that IL-6 play a pivotal role in prostate cancer progression. Thus, levels of IL-6 correlate with disease burden and prognosis and are linked to activation of androgen receptors in androgen-deprived and androgen-independent prostate cancers. (Hobisch et al., 1998; Adler et al., 1999). AT-1 cells are androgen-independent, thus the lack of effects of IL-6 in the study presented herein (paper V) may be due to a masking effect by endogenous IL-6 production and autocrine and/or paracrine signaling.

Lactic acid-induced low pH had little effect upon AEA metabolising enzymes. Exposure to lactic acid may however alter 2-AG homeostasis indicated by increased expression of DAGLα. 2-AG may also exert a more important role in AT-1 cell function considering the sensitivity towards orlistat in the LDH assay. As a caveat, orlistat is a general lipase inhibitor and inhibits DAGLα/β at a low concentration. Thus, it cannot be ruled out that the effect upon cell survival might be mediated via other lipases especially considering that effect was only seen at the highest
concentration (20 μM). TNFα treatment of DU145 cells reduced DAGLα and β mRNA expression suggestive of lower 2-AG levels.

Taken together, all inflammatory factors except IL-6 affected at least one enzymatic player within the eCB system. Although no clear functional consequences of these changes have been reported in this thesis, the data are consistent with the contention that abnormal eCB signaling seen in prostate cancer could be mediated by the tumour environment.

**Future perspectives**

In conclusion, the results presented in this thesis suggest that I) the metabolites of ibuprofen and flurbiprofen have lower FAAH and COX inhibitory potencies than the parent compounds, but in some cases retain their substrate-selective inhibitory properties; II) the chiral center of ibu-AM5 is more important than that of flu-AM1 with respect to FAAH inhibitory potency; III) COX-2 is only a minor contributor to AEA degradation in mouse leukemic macrophage RAW264.7 cells, and IV) inflammatory stimuli and lactate-induced pH changes affect the metabolic enzymes involved in 2-AG turnover.

Possible future studies arising from these findings include the following:

- With respect to the effects of inflammatory factors upon 2-AG related enzymes in prostate cancer: Are the changes in mRNA matched by corresponding changes in enzyme levels, activities, and in levels of 2-AG and related monoacylglycerols?

- With respect to COX-2 as a minor contributor to AEA degradation in the studied cells: what is the biological relevance and effects of prostamidines and prostaglandin glycerols?

- With respect to the dual-target approach: consider other targets for dual-target inhibitors in combination with FAAH, not least NAAA? NAAA hydrolyses NAEs, predominantly PEA that exerts anti-inflammatory effects.
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Time to be sentimental. It is not what I do best, most certain I´ll start to cry. Still, I want to say...

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References


Appendix